UNIVERSITY OF ALBERTA

INVESTIGATION OF DNA SEQUENCES UNIQUE TO *MYCOBACTERIUM BOVIS*

BY

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

DEPARTMENT OF MEDICAL MICROBIOLOGY AND IMMUNOLOGY

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Trust in the Lord with all your heart and
Lean not on your own understanding;
In all your ways acknowledge Him and
He will make your path straight.

Proverbs 3:5-6

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ABSTRACT

We investigated subtractive hybridization as a technique to compare genomic DNA fragments from two mycobacterial strains: *Mycobacterium tuberculosis* H_{37}Ra and a *Mycobacterium bovis* clinical isolate.

Initially, a model plasmid system comparing plasmid DNA with and without an insertion sequence was used to validate the subtraction method. Biotinylated driver DNA was mixed with Digoxigenin labeled target DNA. Four successive subtractive hybridizations removed the backbone DNA common to both plasmid populations, leaving insert DNA fragments unique to the one plasmid population in the reaction mixture. These unique fragments were verified via Southern hybridizations.

Repeated subtractive hybridization between the two mycobacterial strains reduced the DNA content to below that detectable by gel electrophoresis and ethidium bromide staining. PCR amplification of the reaction mixture and agarose gel eletrophoresis resulted in ten visible products which were individually labeled with Digoxigenin and gel purified. In Southern hybridization experiments, all ten fragments found complementary sequences in both the *Mycobacterium tuberculosis* and *Mycobacterium bovis* strains. Four of the fragments produced different banding patterns, and these are considered the most promising as potential unique *Mycobacterium bovis* sequences.

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Who finds a thought that enables him to obtain a slightly deeper glimpse into the eternal secrets of nature has been given great grace.

A. Einstein

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1.0 INTRODUCTION

Throughout the development of classification systems, taxonomists have focused on determining the degree of similarity between organisms. Initially, visible characteristics were sufficient to begin grouping like organisms together. As analytical techniques became more sophisticated, subtle differences within those groups became evident, and molecular biology now provides the opportunity to investigate genetic diversity between highly similar organisms.

Tuberculosis is an infectious bacterial disease caused by members of the tuberculosis complex. This group of four closely related species exhibits 75-100% DNA homology (Imaeda, 1985; Grosskinsky *et al* 1989; Kusunoki *et al* 1991). Of these four, *Mycobacterium tuberculosis* and *Mycobacterium bovis* both cause tuberculosis in mammals. These two organisms were originally named according to their suspected host, leading to misunderstandings. Although *Mycobacterium tuberculosis* is primarily pathogenic to humans, primates and dogs, *Mycobacterium bovis* has a wider host range, causing disease in humans, primates, ruminants, swine, dogs, cats and possibly some birds (Runyon *et al* 1974). To identify the particular causative organism, diagnostic laboratories rely on culturing and biochemical analysis when testing clinical samples.

The culturing of mycobacterial species was first reported by Robert Koch in 1882 (Collins *et al* 1983) and subsequent health policies focussed on elimination of the disease in humans and cattle. However, the resurgence of this pathogen (Tenover *et al* 1993; Bloom *et al* 1992; Drobniewski *et al* 1995) has intensified the need for rapid diagnostic tests which characterize the specific

organism.

All members of the tuberculosis complex have optimal generation times of 14-15 hours (Runyon *et al* 1974) and initial culture and presumptive identification can take several weeks. Drug susceptibility tests require another growth step, adding an additional 2 to 8 weeks for complete characterization. This situation provides an opportunity for the development of diagnostic procedures based on molecular techniques.

Biochemical tests are able to differentiate these two species (Kent *et al* 1985) and these phenotypic differences may reflect a genetic difference. A DNA based test able to target sequences unique to only one of the two organisms would be a rapid diagnostic tool. The challenge is to identify those unique sequences by comparing genomic DNA from each organism. In the study reported here, subtractive hybridization was investigated as a technique to identify unique *M bovis* DNA sequences.

2.0 LITERATURE REVIEW

2.1 Classification of organisms

Organisms of the same species are able to interbreed and produce viable, fertile offspring. The basis for this classification was first proposed by Carolus Linnaeus in the 1700s. Observable characteristics such as motility, structural features and photosynthetic ability allowed him to classify plants and animals by assigning a genus and species name to each living organism.(Lim, 1989)

The discovery of microorganisms added a new challenge for taxonomists, since these living organisms were able to metabolize, grow and reproduce independently from each other. Phenotypic characteristics were the criteria for separating microorganisms into their various species. Within a particular species, strains could be detected, each of which exhibited certain details, usually reflecting derivation from a common ancestor. With improvements in microscopy and biochemical techniques, microbial taxonomists continued to adapt and revise the classification systems. (Lim, 1989)

Molecular techniques provide the opportunity to investigate the genetic basis contributing to the phenotypic characteristics. Organisms of the same species will have highly similar genotypes compared to organisms of different species. Techniques such as nucleic acid hybridization studies, thermal denaturation to calculate G+C/A+T ratios and direct sequencing of DNA and RNA segments all contribute to the understanding of how closely organisms are

related. (Lim, 1989)

The classification of organisms has never been an easy task and new evidence is continually being presented to adjust and move species boundaries. The members of the genus *Mycobacterium* are a good example of the complexity of the taxonomic challenge.

2.2 Mycobacteria

The family *Mycobacteriaceae* is made up of one genus - *Mycobacterium* (Nolte *et al* 1995). Members of this genus are slightly curved or straight bacilli, nonmotile and nonsporeforming. They are considered gram positive, although they are not readily gram stained. The high lipid content of their cell wall impedes uptake of the dye and once stained, resists decolorization, a characteristic known as acid-fastness. The genus contains a natural division between rapid and slow growing organisms based on the time required for colony formation on solid media. Growth rate, pigmentation in the presence and absence of light and colony morphology vary for the different members of this genus (Nolte *et al* 1995).

2.2.1 Tuberculosis complex

2.2.1.1 Members

The *Mycobacterium tuberculosis* complex includes the four closely related

species of *Mycobacterium tuberculosis (M tuberculosis), Mycobacterium bovis (M bovis), Mycobacterium africanum (M africanum)* and *Mycobacterium microti (M microti)*. These are all slow growing organisms, pathogenic for various hosts. *M tuberculosis* produces tuberculosis in man, other primates and dogs. Experimentally, it is pathogenic for guinea pigs and hamsters, and relatively nonpathogenic for rabbits, cats, goats, bovine animals and domestic fowl. *M bovis* produces tuberculosis in cattle, domestic and wild ruminants, man and other primates, dogs, cats, swine, parrots and possibly some birds of prey. *M. bovis* is generally more pathogenic for animals than is *M tuberculosis*. *M africanum* is an interesting human isolate found in tropical Africa. *M microti* is pathogenic only to voles and may represent an intermediate form between *M tuberculosis* and *M bovis*. The bacillus of Calmette-Guerin (BCG) is an attenuated *M bovis* isolate, and has been extensively used as a tuberculosis vaccine in many parts of the world. It exhibits most of the characteristics described for *M bovis*, although it is less pathogenic.(Runyon *et al* 1974)

2.2.1.2 Genetic relatedness

Although similar phenotypes suggest closely related organisms, true relatedness will be evident in genetic analyses and various techniques have been used to determine the genetic relatedness of these four organisms.

2.2.1.2.1 DNA homology studies

The general principle to determine the percent DNA homology between two bacterial species involves purification of DNA from two sources, complete denaturation and hybridization of the two populations to each other. The amount of cross-hybridization indicates the degree of homology between the two species.

Gross and Wayne (1970) performed nucleic acid homology studies using Oxoid Ionagar columns. When compared to the *M tuberculosis* reference, relative percent binding for the three *M tuberculosis* strains was 78.9 to 108%.

DNA filter hybridization studies reported by Grosskinsky *et al* (1989) showed the tuberculosis complex samples analysed by this method were between 75 and 100% homologous.

Kusunoki (1991) developed a microdilution plate hybridization technique to identify mycobacterial species. Their results showed a relative similarity between *M tuberculosis* and *M bovis* of 95 to 100% and these two organisms could not be differentiated by this method. The author suggests reclassifying these as one species differentiated as pathovars or biovars.

Other hybridization studies report similar results. Imaeda (1985) investigated selected strains from all four species in the tuberculosis complex and found between 85 and 100% DNA relatedness. Bradley (1973) used reassociation techniques to investigate mycobacterial strains, including one avirulent *M tuberculosis* strain ($H_{37}Ra$) and one *M bovis* BCG strain. Their optical reassociation tests showed that *M bovis* was 86% homologous with

$H_{37}Ra$.

Although these DNA-DNA hybridization techniques can directly compare two populations of genomic DNA, the results can be misleading. These tests may not detect certain unmatched regions such as deletions, insertions or areas of base substitutions. Initial standardization can be time consuming and the literature describes a variety of optimal conditions, leading to difficulty in comparing results.

2.2.1.2.2 DNA sequence analysis

Comparison of ribosomal RNA gene sequences and restriction endonuclease (RE) analysis indicate the high homology between organisms.

Slow growing mycobacteria have a single ribosomal RNA operon (Kempsell *et al* 1992). Glennon *et al* (1994) found 100% sequence homology in the 16S to 23S ribosomal DNA internal transcribed spacer (ITS) region of type strains representing all the members of the tuberculosis complex. These results were substantiated by Frothingham *et al* (1994).

Restriction endonuclease (RE) analysis identifies the location of RE recognition sites in chromosomal DNA (Eisenach *et al* 1986). When the chromosomal DNA is digested and the fragments are electrophoresed, a banding pattern is seen that is specific for a particular strain. In other strains, slight differences in the DNA sequence of the RE recognition site will produce a different pattern.

An initial study done by Collins and De Lisle (Collins *et al* 1984) involved

restriction endonuclease analysis of various *M tuberculosis* strains and one BCG isolate. Analysis of the fragment patterns showed a very high similarity, some strains being indistinguishable from each other.

In another report, Collins and De Lisle (1985) compared clinical isolates and reference strains from all 4 species of the tuberculosis complex. Their study found very few differences in the DNA of the various strains. These authors concur with earlier studies which suggest *M tuberculosis* and *M bovis* are closely related yet distinct organisms.

Eisenach *et al* (1986) used cloned DNA probes to analyze restriction endonuclease digests of reference strains of *M bovis* and *M bovis* BCG along with clinical and reference strains of *M tuberculosis*. They were not able to quantitate the similarities and differences. However, they did conclude that some regions of the mycobacterial chromosome are highly conserved, while others demonstrate more heterogeneity.

Although these RE profiles can identify changes in certain enzyme recognition sites, they are technically demanding, requiring long gels and extended gel running times. The enzyme used to digest the genomic DNA will change the banding pattern and this choice varies from lab to lab. The intricacy of the patterns can make analysis difficult and intensive. Resolution can also be a problem with these large gels since clarity in one area of the gel is obtained at the expense of other areas.

2.2.1.2.3 G+C content and genomic molecular weight

G+C content is estimated using thermal denaturation measurements, which are based on the stability of the three hydrogen bonds between guanine and cytosine bases versus only two bonds between the adenine and thymine bases. Under comparable conditions, a DNA strand with a majority of G and C bases will remain double stranded at a higher temperature than a DNA strand of the same length containing primarily A and T bases. Samples of DNA can be assayed by slowly raising the temperature and measuring the increase in single stranded DNA present as the double strands dissociate.

Genomic molecular weights are calculated from DNA reassociation values since following denaturation, small simple genomes will reassociate faster than more complex genomic populations.

Imaeda (1985) reported that all four tuberculosis complex members showed mol%G+C between 62.3 and 63.3%, and genomic molecular weights between 1.9 and $2.3X10^9$ daltons. Bradley (1973) used optical reassociation to investigate 11 mycobacterial strains. The one avirulent *M tuberculosis* strain ($H_{37}Ra$) was $2.5X10^9$ daltons and the one *M bovis* BCG strain was calculated to be $2.8X10^9$ daltons.

With these dissociation and reassociation tests, extensive controls are required to regulate the process and ensure accurate measurements. Baess (1984) used the *E coli* genome as a standard and calculated the genome size for three of the tuberculosis complex organisms as ranging from 2.34 to $2.73X10^9$ daltons. *M bovis* samples were not included in this study, however, *M bovis*

BCG was tested. The G+C content for all samples ranged from 64.9 to 71.4%, agreeing with other published data. This work has been further investigated by Aoki and Yamada (1994) as a potential diagnostic procedure, but still does not differentiate within the complex.

2.3 Tuberculosis control programs

Differentiation of pathogenic mycobacterial species is critical to national tuberculosis control programs. For nearly a century, programs have focussed on protecting the health of two interrelated populations, humans and domesticated animals.

In 1882, when Robert Koch made a public announcement that he had cultured the organism responsible for causing tuberculosis (Collins *et al* 1983), the disease was a major cause of mortality in humans and animals. Since then, control programs in developed countries have reduced the prevalence of tuberculosis, to the point where some health officials look forward to complete eradication of the disease (Anonymous, 1990). The progress of these programs was a reflection of the methods developed to identify specific characteristics of the organism responsible for the disease.

2.3.1 Tuberculosis in cattle

Tuberculosis in cattle is caused by transmission of *M. bovis*, primarily via the airborne route (Morris *et al* 1994). Inhalation of *M bovis* in droplet nuclei

results in lesions occurring in the lungs and associated lymph nodes (Neill *et al* 1994). Infected animals are detected using purified protein derivative tuberculin in an intradermal skin test (Monaghan *et al* 1994).

Wildlife reservoirs seem to be a continuing source of infection, but this depends on the country studied and their tuberculosis status (Neill *et al* 1994). Transmission of *M bovis* from human to cattle has been documented (Hardie *et al* 1992; Grange *et al* 1994) and is considered a grave risk (Collins *et al* 1983; Kovalyov, 1989). In contrast, transmission of *M tuberculosis* from human to cattle is limited and natural infection in cattle from *M tuberculosis* does not generally lead to progressive disease (Hardie *et al* 1992).

In several developed countries eradication of tuberculosis in cattle has been a focus of health and agricultural officials (Hardie *et al* 1992; Kovalyov, 1989; Pritchard, 1988; Robinson *et al* 1988; Mylrea, 1990; Wigle *et al* 1972; Caffrey, 1994; Essey *et al* 1994). Accrediting programs were established based on intensive herd testing and removal of animals. In Canada, as in other countries, these efforts resulted in cattle herds which are internationally valued for their tuberculosis-free status (Caffrey, 1994; Essey *et al* 1994). The focus now is to continue such monitoring programs and identify *M bovis* infections as rapidly as possible.

2.3.2 Tuberculosis in humans

Pulmonary tuberculosis in humans can result from inhalation of aerosolized droplets containing either *M tuberculosis*, *M bovis* or *M africanum*.

With the increase in immunocompromising conditions such as HIV infection, homelessness, urban overcrowding and poor health care, the incidence of human tuberculosis has begun a resurgence (Tenover *et al* 1993; Alland *et al* 1994; Daborn *et al* 1993; Bloom *et al* 1992; Drobniewski *et al* 1995). *M bovis* is the causative agent in 1 to 6% of isolates from human tuberculosis patients, and several reports describe either reactivation of previous infections or development of disease after exposure to diseased cattle and their products (Kovalyov, 1989; Sauret *et al* 1992; Glennon *et al* 1994; Robinson *et al* 1988; Hardie *et al* 1992). Identification of the causative organism is essential for effective diagnosis and treatment.

Treatment of human tuberculosis now requires multiple drugs over an extended period of time (Nolte *et al* 1995). Brausch and Bass (1993) review the development of antituberculosis drugs and discuss the emergence of drug-resistant organisms in patients on treatment or during subsequent relapse. They report that "more than one agent to which the bacilli is sensitive is necessary to prevent resistance, and strong initial therapy with at least three effective drugs is necessary for short-course chemotherapy (6 months) to be successful". Researchers in several countries report the occurrence of multiple drug-resistant tuberculosis isolates (Morris *et al* 1995; Williams *et al* 1994; Tenover *et al* 1993) and rapid drug susceptibility information is important for effective therapy. Pyrazinamide is a first line antituberculosis drug (Nolte *et al* 1995). Since *M bovis* is naturally resistant to pyrazinamide (Inderlied, 1991), identifying the causative agent as *M bovis* allows clinicians the opportunity to select the proper drug regime.

Present tuberculosis control programs rely on laboratory techniques to correctly identify the infectious organism and detail its specific characteristics.

2.4 Detection of tuberculosis complex organisms

The combination of acid-fast staining, microscopy and culture forms the basis for mycobacterial diagnosis (Drobniewski *et al* 1994; Ford *et al* 1994) and accurate identification requires a combination of test results. Initial tests relied on phenotypic characteristics as the criteria for sorting out the various species. As molecular techniques emerged, tests improved by identifying the genetic basis for those phenotypic differences.

2.4.1 Phenotype-based tests

2.4.1.1 Biochemical tests

There are at least 15 standard biochemical tests available to distinguish mycobacterial species and Kent and Kubica (1985) provide detailed information about four tests which can differentiate *M tuberculosis* from *M bovis*.

The niacin test is the most commonly used test for *M tuberculosis* (Gross *et al* 1985). All mycobacterial species produce niacin. Since *M tuberculosis* lacks the next enzyme in the biochemical pathway, niacin accumulates in the media and can be detected. With some *M bovis* strains, niacin accumulation may be detected.

Reduction of nitrate is catalyzed by the enzyme nitroreductase. *M tuberculosis* produces this enzyme and reduces nitrates to nitrites, while *M bovis* does not. Biochemical tests check for the activity of this enzyme by adding nitrates to the media.

M tuberculosis is able to grow in the presence of thiophen-2-carboxylic acid hydrazide (TCH) while *M bovis* is susceptible to low concentrations of this chemical. Some isoniazid resistant strains of *M bovis* may be resistant to TCH.

The antibiotic pyrazinamide can be included in the media and will inhibit the growth of *M tuberculosis*. Pyrazinamidase is the enzyme in *M tuberculosis* that is responsible for converting pyrazinamide into the active agent pyrazinoic acid. Since *M bovis* lacks this enzyme, the pyrazinamide remains inactive and the organism grows uninhibited (Inderlied, 1991).

Definitive diagnosis of *M tuberculosis* or *M bovis* can be obtained by combining these test results with such information as case history and organism growth characteristics. These tests are performed on actively growing pure cultures and can take anywhere from 2 to 8 weeks depending on the original sample condition and the growth media (Drobniewski *et al* 1994). The main reason for this delay is the slow growth of these organisms. Under optimal condition, the generation time for *M tuberculosis* is 14-15 hours, and the type of media and size of the inoculum will affect the growth rate (Runyon *et al* 1974). Data on drug susceptibility and inhibition of growth can take a further 2-8 weeks (Drobniewski *et al* 1994; Crawford, 1994). Accuracy of the tests may be affected by the culture age, reaction temperature, presence of enzyme inhibitors or contamination by other bacterial species.

2.4.1.2 Immunodiagnostic detection methods

Enzyme linked immunosorbent assays (ELISAs) may be applicable for mycobacterial diagnosis, especially with tuberculosis meningitis (Ford *et al* 1994). Two different versions of this assay can be used for the detection of either mycobacterial antigens present in clinical samples or antimycobacterial antibodies present in patient sera and other body fluids.

In the detection of mycobacterial antigens, the source of the test sample is important (Ford *et al* 1994). Patient sputum samples are routinely treated before testing to remove non-mycobacterial organisms, and these decontamination steps may denature or remove soluble antigens from the sample. This ELISA method has been effective in detecting low levels of mycobacterial antigens in cerebrospinal fluid samples (Radhakrishnan *et al* 1991), possibly because these samples are not decontaminated before processing.

Purified mycobacterial antigens can be used in ELISAs to test for the presence of specific antibodies in patient sera. The challenge with this type of test development is the specificity of the initial antigen and several assays have been developed that are based on different mycobacterial antigens (Daniel *et al* 1987).

Purified antigens and monoclonal antibodies may prove applicable as a diagnostic test for tuberculosis in cattle. Fifis *et al* (1994) used interferon-γ assays and lymphocyte proliferation assays to measure the cellular response of experimentally infected cattle. Variations in the immune response of each

infected animal suggested to these authors that a combination of several antigens may be required.

These immunodiagnostic methods exhibit substantial variability in results (Ford *et al* 1994; Kallenius *et al* 1994). Sensitivity and specificity depend on the degree of infection in the test population, the antigen used and the types of control populations studied. These characteristics limit the practicality of these methods (Kallenius *et al* 1994).

2.4.1.3 Phage typing

Phage typing is a tool for differentiating *M tuberculosis* isolates. Snider *et al* (1984) worked with pure *M tuberculosis* cultures and identified 8 major phage types, subdividing each with 6 auxiliary typing phages. They found no diagnostic advantage to using this technique since it is time-consuming, labour-intensive and requires technical expertise. They suggest its value during epidemiological investigations and suspected contamination of diagnostic and therapeutic equipment.

2.4.1.4 HPLC

High molecular weight long chain mycolic acids are a component in the cell wall of all mycobacterial species (Jones *et al* 1986). Following extraction from cell wall suspensions, mycolic acids can be separated by high performance liquid chromatography (HPLC) and early studies of this technique began

identifying unique patterns for the different mycobacterial species (Butler *et al* 1986; Butler *et al* 1988). However, recent studies are only able to differentiate *M bovis* BCG isolates from the other tuberculosis complex organisms (Butler *et al* 1991; Thibert *et al* 1993). Glickman *et al* (1994) developed a computer based mycobacterial file containing various mycolic acid HPLC patterns. Without this system, they found that interpreting the HPLC data required some expertise for the visual assessment and manual calculations. With a database full of previously known patterns, this program could quickly analyse unknown isolates and report results. In listing the strains used to construct their computer database, *M tuberculosis* and *M bovis* BCG are the only two for the tuberculosis complex, indicating that *M bovis* and *M africanum* would not be distinguished from *M tuberculosis*.

2.4.1.5 Radiometric

The radiometric BACTEC system allows for shorter isolation time and presumptive identification of mycobacteria (Drobniewski *et al* 1994). The assay detects $^{14}CO_2$ produced by mycobacteria during the metabolism of ^{14}C labeled palmitic acid (Drobniewski *et al* 1994). Vials of liquid media are inoculated with decontaminated patient specimens and incubated, with readings automatically taken at preset intervals, usually 1-3 times per week. When the growth index reaches a certain level, aliquots of the culture can be used for further study, even though the culture is not at the visible growth stage. Ziehl-Nielsen staining will confirm the presence of acid-fast bacteria and check for cellular morphology

consistent with members of the tuberculosis complex. Inoculation of the media with p-nitro-α-acetylamino-β-hydroxypropiophenone (NAP) will identify tuberculosis complex organisms since these are inhibited by NAP, while other mycobacterial species are unaffected (Morgan *et al* 1985). BACTEC media containing thiophene-2-carboxylic acid hydrazide (TCH) will differentiate cultures of *M tuberculosis* from *M bovis* since *M bovis* strains are usually inhibited by TCH.

Gross and Hawking (1985) used these radiometric techniques to differentiate *M tuberculosis*, *M bovis* and non-tuberculosis complex organisms from each other. Pure cultures were used to inoculate BACTEC vials containing either NAP or TCH. Their results were consistent with conventional test results, but they were able to provide the results within 5 days of receiving the cultures, compared to conventional tests which can take weeks.

2.4.2 Genotype-based tests

In medical diagnostic labs, microbiologists can detect and identify most clinically significant infectious organisms within 24 hours (McFadden *et al* 1990). However, with the slow growing mycobacteria, molecular techniques are potentially faster identification methods.

2.4.2.1 Probes

The specificity of nucleic acid base pairing means that DNA probes are

able to detect unique portions of nucleic acids that are carried by infectious organisms (Pfaller, 1994). Specific probes are commercially available and many more have been reported by research labs.

Gen-Probe Inc (San Diego, California, USA) marketed the first commercial DNA probes for mycobacterial detection. Their first kit was an hybridization test which uses a specific ^{125}I-DNA probe complementary to the ribosomal RNA sequence of tuberculosis complex organisms (McFadden *et al* 1990). The system can provide a savings in time of 3-8 weeks (McFadden *et al* 1990), however, the radioactivity hazard and the short half life of the probe (60 days) were drawbacks. As well, further tests are required to identify individual complex members.

The discovery of repeated insertional DNA elements in *M tuberculosis* has improved probe sensitivity. Poulet and Cole (1995) reviewed five sequences specific to the tuberculosis complex. IS*6110* was the first repeat sequence characterized and has been studied most intensively (Poulet *et al* 1995). This element had only been found in tuberculosis complex organisms, where it was present in 6 to 17 copies in *M tuberculosis* and *M africanum*, 1 to 5 copies in *M bovis* and as a single copy in *M bovis* BCG. Initially, these characteristic copy numbers offered a definite way to separate and identify the complex members. Recent discovery of *M tuberculosis* strains with few or no copies of the IS*6110* (van Soolingen *et al* 1993) has cautioned researchers. IS*1081* was initially found in *M bovis* and is specific for the tuberculosis complex. The major polymorphic tandem repeats (MPTR) which consist of 10 bp direct repeats separated by 5 bp unique segments have been detected in species

other than the tuberculosis complex. This element shows only slight discrimination when used to test *M tuberculosis* isolates (van Soolingen *et al* 1993). The polymorphic GC rich repetitive sequence (PGRS) shows potential for epidemiological studies. Van Soolingen's study (1993) involved *M tuberculosis* isolates from seven different countries and found that PGRS was an excellent marker for use in differentiating *M tuberculosis* strains. His work did not include other members of the tuberculosis complex. A 36 bp direct repeat (DR) cluster is present at a single chromosome locus and van Soolingen (1993) reported that it was very polymorphic, distinguishing the 63 *M tuberculosis* strains studied.

As with other potential diagnostic tools, it seems that no single repeat sequence is able to definitely distinguish members of the tuberculosis complex. These repeat sequences were investigated because they provided researchers with a naturally amplified target DNA sequence, meaning the assay would be more sensitive. The polymerase chain reaction (PCR) is another way to amplify DNA and provide more target for probe hybridization.

2.4.2.2 PCR

In 1988, Saiki *et al* (1988) reported the use of a thermostable DNA polymerase in the polymerase chain reaction (PCR). The use of this heat resistant enzyme in PCR led to a dramatic increase in the reaction's specificity, yield, sensitivity and length of amplifiable targets. The reaction is a series of cycles which result in the exponential amplification of a specific segment of

target DNA and is valuable whenever there is a limited amount of the target. To verify the amplification of the proper target sequence, reaction products were transferred from an agarose gel onto membranes and hybridized with a labeled probe specific for the target DNA sequence.

PCR has been successful in amplifying *M tuberculosis* from clinical isolates (Sritharan *et al* 1994; Pietrzak *et al* 1994; Nolte *et al* 1993). These studies used primers and probes designed to detect the repetitive sequence IS*6110*. Although using a repetitive sequence provides more target DNA per bacterial cell and therefore increases the sensitivity of the PCR, all members of the tuberculosis complex contain these sequences and will not be differentiated.

Del Portillo *et al* (1991) developed a test to differentiate *M tuberculosis* from *M bovis*. She PCR amplified a 369 bp fragment from uncultured clinical samples which appears to be specific for *M tuberculosis* since it was not present in the other mycobacterial and non-mycobacterial species tested, including *M bovis* and *M bovis* BCG. The target sequence is present as a single copy and a combination of PCR and probe hybridization was able to detect 10 fg of DNA. Similarly, Patel *et al* (1990) used American Type Culture Collection strains and differentiated *M tuberculosis* H_{37}Rv from *M bovis* based on amplification of a DNA sequence specific to H_{37}Rv and *M bovis* BCG. PCR products from as little as 1 fg of target DNA were visible on agarose gels.

The speed and sensitivity of PCR makes a species-specific procedure very attractive to mycobacteriologists. Genus- and complex-specific PCR tests may prove valuable since initial PCR screening would identify positive samples.

2.4.2.3 RFLP

Restriction fragment length polymorphisms (RFLP) can identify sequence differences between two samples. Genomic DNA is digested by restriction enzymes (RE) and the products are electrophoresed through an agarose gel. The fragments are transferred to a membrane and a labelled probe hybridizes to complementary sequences throughout the DNA sample. The specificity of the hybridization pattern is valuable for epidemiological studies when relatively stable elements are used as probes (Hermans *et al* 1990; van Soolingen *et al* 1991; van Soolingen *et al* 1994; van Embden *et al* 1993). Varying the probe sequence or using a different enzyme will produce different levels of pattern intricacy. When the probe is targeted towards a repetitive sequence, the polymorphic pattern becomes even more intricate and may differentiate closely related strains.

IS*6110* is a repetitive sequence that has been studied so intensively that van Embden *et al* (1993) proposed a standardized methodology for RFLP strain typing based on this element. However, the IS*6110* does not produce banding patterns which consistently differentiate the individual members of the tuberculosis complex. Van Soolingen *et al* (1991) used the IS*986* element to analyse 278 clinical isolates representing all members of the tuberculosis complex. This insertion sequence is virtually identical to IS*6110* (Hermans *et al* 1990), which explains why van Soolingen *et al* (1991) focussed on comparing RFLPs within each species, rather than between the four complex members.

Pfaller (1994) makes the comment that DNA based assays should "detect

and identify an infectious organism directly in the clinical sample with the sensitivity of culture, but in days rather than weeks" (pg.330). Effective diagnostic tests using molecular techniques will be based on a fragment of DNA that is unique to the organism of interest.

2.5 Identifying unique fragments

2.5.1 Differential screening

Figure 1 illustrates differential screening. This strategy has successfully identified unique clones in eucaryotic (Tedder *et al* 1988) and procaryotic DNA libraries (Patel *et al* 1989). Patel *et al* (1989) searched for unique *M tuberculosis* H_{37}Rv DNA fragments by probing duplicate filters with either nick translated *M tuberculosis* H_{37}Rv DNA, or a labeled mixture of non-tuberculosis complex DNA. Out of more than 1,000 clones, three were specific for tuberculosis complex organisms.

There are three advantages to this strategy. It is possible to screen a large number of clones simultaneously, positive sequences are already cloned, and these clones are easily isolated and prepared for further manipulations such as sequencing or expression.

One disadvantage to this procedure is the need for complete genomic libraries. As the homology between the two organisms increases, the libraries will contain fewer unique segments and it is very important that these segments are included in the original libraries. A second concern is that rare sequences

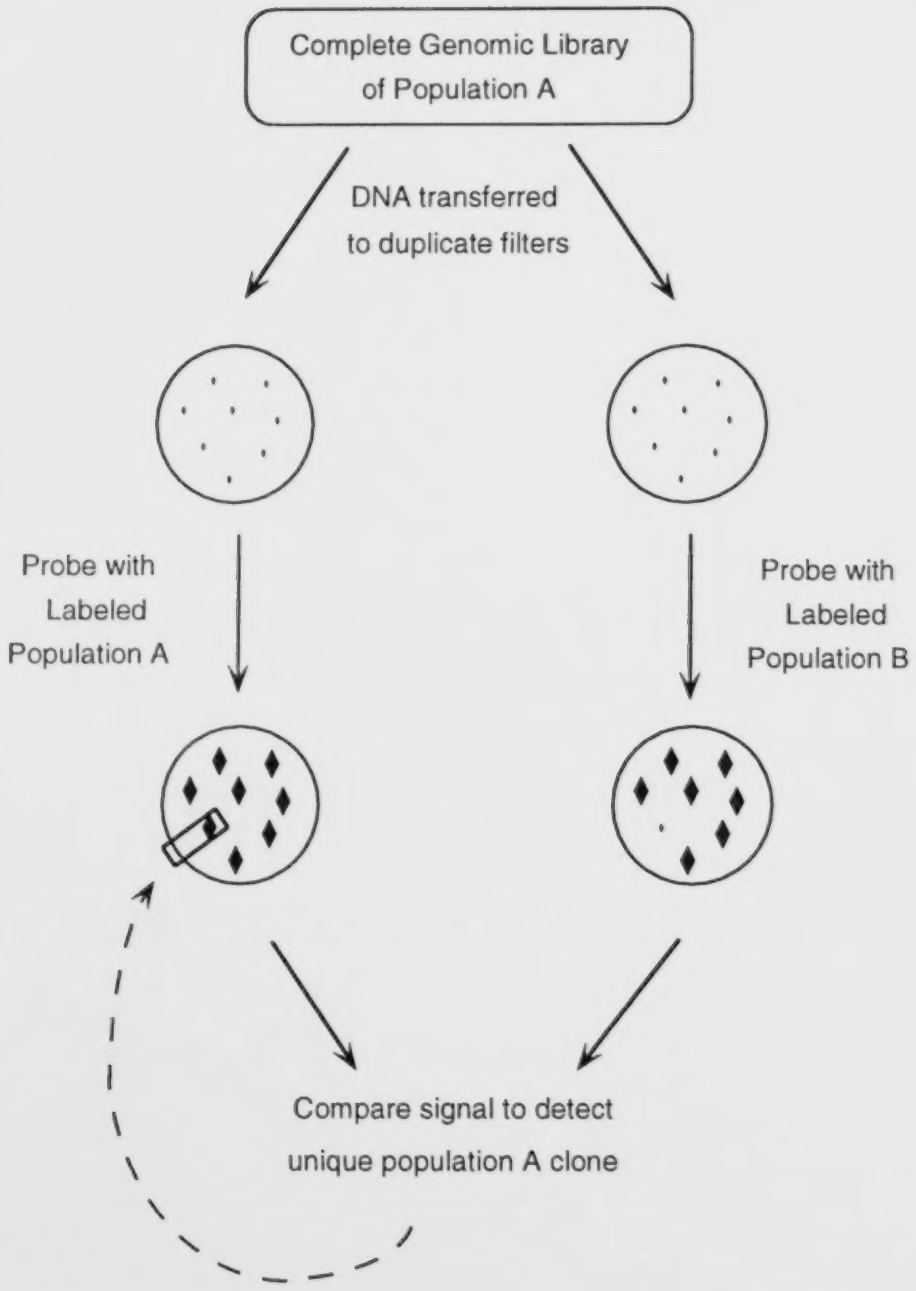


Figure 1. Differential screening scheme.

will be at a low concentration in the probe mixture in relation to the rest of the labeled fragments, and may have difficulty hybridizing to a level that can be detected (Klickstein, 1992). An alternative to differential screening is subtractive hybridization.

2.5.2 Subtractive hybridization

Subtractive hybridization is designed to differentiate between two closely related nucleic acid populations by selecting for a fragment which is present in one population and absent in the other. As illustrated in Figure 2 the first step is purification of DNA or mRNA from the two different sources. The samples can be labeled for future identification before they are combined, denatured and allowed to hybridize. With related organisms, the majority of DNA is identical and cross-hybridization will occur between the two populations. Fragments unique to one population will not find complementary sequences in the other population, and will reanneal to their own complementary sequences. When the common fragments are removed, the unique fragments remain and are available for resubtraction or analysis.

The use of subtractive hybridization to isolate a deleted DNA sequence was first reported by Bautz and Reilly (1966). Their approach was to compare the DNA from a mutant and a wildtype bacteriophage strain. Their experiments were time-consuming and detailed, purifying a small segment of the wildtype DNA which had been deleted from the mutant genome.

Since these first experiments, subtractive hybridization has been adapted

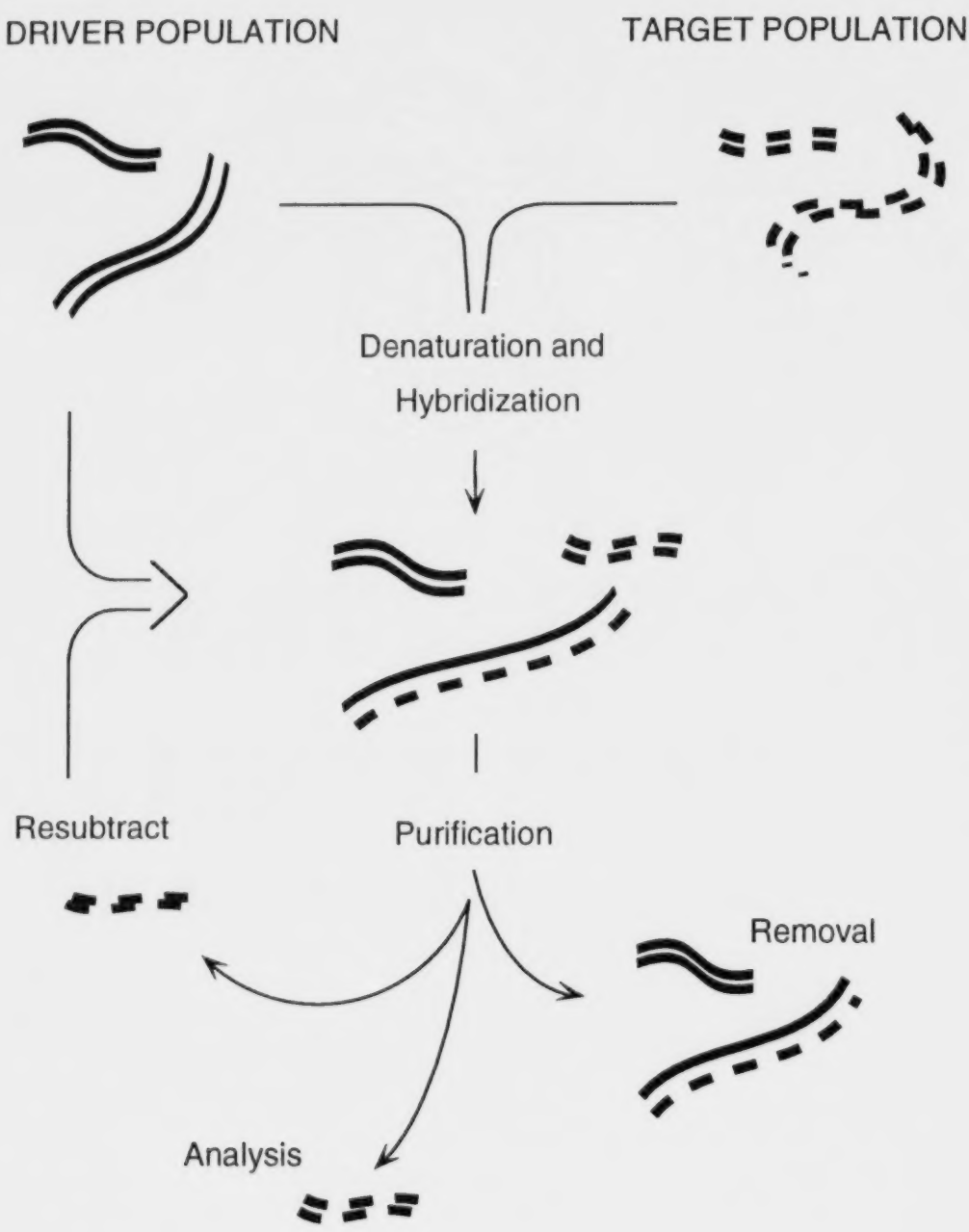


Figure 2. Subtractive hybridization scheme.

and extensively used to understand a variety of genetic alterations. Advances in cloning strategies and the ability to amplify DNA fragments via PCR have enhanced the procedure.

2.5.2.1 Applications

Subtractive hybridization has been applied to eucaryotic systems. The following three examples describe the identification of deletions in the Y chromosome, deletions in the X chromosome and identification of cell specific mRNA fragments.

Lamar and Palmer (1984) used a 'deletion enrichment' strategy to clone Y chromosomal DNA. They used inbred mice and hybridized an excess of sheared female DNA with *Mbo* I digested male DNA. The Y chromosomal DNA fragments self-annealed and were ligated into an appropriate vector for cloning. The resulting library was screened and 13% of the inserts were Y specific DNA.

A similar technique was reported by Kunkel *et al* (1985) when they cloned probes for the Duchenne muscular dystrophy (DMD) syndrome. Their strategy involved hybridization of sheared DNA isolated from a male child with an X chromosome deletion with *Mbo* I digested DNA from a human lymphoid cell line. The unique *Mbo* I fragments were cloned, resulting in a recombinant library enriched for DMD segments absent from the DNA of this child.

A more recent example was reported by Marechal *et al* (1993). Rather than comparing chromosomal DNA from two organisms, they looked at mRNA populations from different organs in the same organism. Peripheral mRNA

samples from rat liver, kidney, spleen and intestines were immobilized on nitrocellulose membranes. Single stranded labeled cDNA was synthesized from purified rat brain poly(A)+ mRNA, and hybridized to peripheral organ mRNA samples. A cDNA library was created using the purified fragments. Fifty five percent of the inserts tested were brain-specific, failing to hybridize with the mRNA samples from other organs.

Subtractive hybridization has also been successfully used to study bacterial genomes, as described in the following four examples.

Seal *et al* (1992) combined excess sheared *Xanthomonas campestris* DNA with *Mbo* I digested *Pseudomonas solanacearum* DNA. One positive clone was selected from the resulting library and proved to be a *P solanacearum* species-specific DNA probe.

Kinger and Tyagi (1993) studied virulence-specific genes of *M tuberculosis*. They hybridized cDNA from a virulent *M tuberculosis* strain ($H_{37}Rv$) with excess RNA from the avirulent $H_{37}Ra$ strain. Rather than an unique DNA fragment, two rounds of subtraction identified differentially expressed genes which require further analysis for virulence-associated function.

Plum and Clark-Curtiss (1994) investigated the genes of *M avium* that are specifically expressed by the bacilli grown in a macrophage cell line, compared to the same strain grown in broth culture. Messenger RNA from each cell line was converted to cDNA and ligated to oligonucleotide adaptors. After three rounds of subtraction the PCR amplified products were used to screen cosmid libraries. One positive clone was highly specific for *M avium* bacilli growing within macrophages.

A similar approach was used by Kikuta-Ashima *et al* (1994). They briefly report on subtraction of $H_{37}Rv$ cDNA with excess $H_{37}Ra$ RNA. The $H_{37}Rv$ products identified fragments in a Southern blot which contain genes expressed in the virulent strain ($H_{37}Rv$) but not the avirulent ($H_{37}Ra$) strain.

2.5.2.2 Technique improvements

Since Bautz and Reilly (1966) first published their work, various techniques have enhanced the subtractive hybridization procedure.

First, biotin is a popular label because of the strong binding of biotin with avidin (Fujita *et al* 1993). Straus and Ausubel (1990) used photoactivatable biotin to label the driver DNA population. Following the subtraction step, biotinylated fragments were removed by adding avidin-coated polystyrene beads and filtering the mixture. Aasheim *et al* (1994) attached streptavidin-coated magnetic beads directly to the biotinylated driver cDNA before the hybridization step. Rodriguez and Chader (1992) describe magnetic beads attached to oligo d(T) fragments which bind mRNA and prime the synthesis of first strand cDNA and this becomes the driver population. With magnetic beads, the subtraction products are placed near a magnet to remove the beads and attached hybrids, leaving the fragments of interest in the supernatant.

Second, cloning strategies have improved the subtraction procedure. For example, target DNA can be digested with specific restriction endonucleases. Following subtraction, the reannealed target fragments can be immediately cloned (Seal *et al* 1992) or ligated with adaptors for PCR amplification (Plum *et*

al 1994; Straus *et al* 1990), regardless of common fragments which may copurify. A second example describes a strategy using single stranded phagemids to compare directional cDNA libraries (Rubenstein *et al* 1990). Representational difference analysis (RDA) is a third example which involves ligation of one 5' adaptor to the target DNA followed by subtractive hybridization and PCR amplification (Lisitsyn *et al* 1993).

Third, subtractive hybridization has been combined with other molecular biology techniques. For example, in-gel competitive reassociation (IGCR) involves subtractive hybridization along with gel electrophoresis (Yokota *et al* 1994). In combination with RFLP, Rosenberg *et al* (1994) used subtractive hybridization to isolate unique sequence markers from the genomic DNA of two inbred mouse strains. Hakvoort *et al* (1994) used subtractive hybridization along with a differential display method, providing an alternate way to analyse the subtraction products and identify underrepresented gene products.

2.5.2.3 Necessary considerations

Researchers have addressed the limitations of the subtractive hybridization technique by analyzing conditions and developing improvements which apply to six general areas.

First, the source and type of nucleic acid must be given careful consideration. The two cell lines must be different enough to ensure a sequence difference, and yet not so distinct that analysis of the many subtraction products is time-consuming. The type of nucleic acid will depend on the research focus.

When the investigation is a difference in gene expression between two cell populations, the choice would be to purify mRNA from each source, converting one sample into cDNA to allow for hybridization between the two populations. To investigate an environmental adaptation or a change in virulence, mRNA from the expressing population would be subtracted with either mRNA or genomic DNA fragments from the non-expressing population. A subtraction procedure that compares DNA from both sources would identify a genetic difference, most often representing a deletion or an insertion.

Second, it is important to critically analyse the nucleic acid purification steps and consider possible sources of contamination (Lisitsyn *et al* 1993). Exogenous DNA in the target sample would be isolated during the subsequent subtraction steps, since these fragments will not find complementary sequences in the driver population. If the two nucleic acid populations are purified, digested or analysed simultaneously, cross contamination would mean the two are no longer distinct.

Third, the DNA samples are usually labeled and/or ligated to adaptors for future capture or amplification. When driver DNA is biotinylated, it is important that each fragment be labeled to allow for complete removal following the subtraction step. Inefficient adaptor ligation means the primers used in the subsequent amplification steps will not bind to the fragment.

Fourth, Straus and Ausubel (1990) discuss the importance of proper hybridization conditions. Short sequences with low annealing temperatures may remain single stranded if the stringency is too high. Conversely, fragments that have palindromic sequences may preferentially self-anneal if stringency is too

low or the sample is too dilute.

The fifth concern is detection of the purified products. Direct cloning of the subtraction products may be preferred when only a few different fragments are suspected, however, common sequences will obscure the results and lengthen the time required to analyse the clones (Hakvoort *et al* 1994). PCR amplification of the subtraction products would select for the target DNA fragments making analysis easier.

Finally, establishing a model subtractive hybridization system with sequenced DNA populations will define the most important parameters of a particular subtraction procedure. Lisitsyn *et al* (1993), van Klaveren *et al* (1994) and Yokata *et al* (1994) describe working with known DNA populations to establish parameters and customize the subtraction steps to meet particular research needs.

3.0 HYPOTHESIS

3.1 Hypothesis

That subtractive hybridization will select for DNA sequences unique to *Mycobacterium bovis*

3.2 Specific Objectives

1. To isolate pure, intact genomic DNA from two mycobacterial strains: a *Mycobacterium tuberculosis* H_{37}Ra laboratory strain and a *Mycobacterium bovis* clinical isolate

2. To test and validate the subtractive hybridization steps using a model system

3. To apply the established subtractive hybridization procedure to the detection of unique DNA sequences in *Mycobacterium bovis*

4.0 MATERIALS AND METHODS

4.1 Plasmids, bacterial strains and media

The two plasmids used in this study were obtained from Dr J.F. Elliott, Medical Microbiology and Immunology, University of Alberta. Figure 3 is a schematic drawing of the plasmids with the relevant restriction enzyme sites. Smith *et al* (1992) give a detailed explanation of the plasmid construction and the insert sequence (GenBank accession number M84025). Briefly, pHAS is a modification of the vector pBluescript KS- and pHAS+I is the same pHAS vector containing a 1543 bp insert (I). From the sequence data, these two plasmids are 65% homologous. Digestion of pHAS with the *Hae* III restriction endonuclease produces 15 fragments: 2 multiple cloning site (MCS) fragments (M) and 13 backbone fragments with the following sizes: 767, 458, 434, 294(M), 267, 243, 174, 170(M), 142, 125, 102, 80, 54, 18 and 11 bp. *Hae* III digestion of the pHAS+I plasmid produces 21 fragments: 6 unique insert fragments (I), 2 partial fragments (part insert and part MCS, P) and 13 backbone fragments (identical to the pHAS backbone fragments) with these sizes: 767, 458, 446(P), 434, 309(I), 267, 262(I), 243, 174, 171(I), 151(P), 142, 139(I), 127(I), 125, 102, 80, 54, 33(I), 18 and 11 bp.

DH5α electroporation cells (Sheen, 1994) were obtained from Lina Cho-Leam, ADRI Lethbridge. SOC media (Sheen, 1994), Superbroth and Luria-Bertani (LB) agar plates (Lech and Brent, 1988) were prepared by the ADRI media lab using products from Difco Laboratories, Detroit, Michigan, Canadian

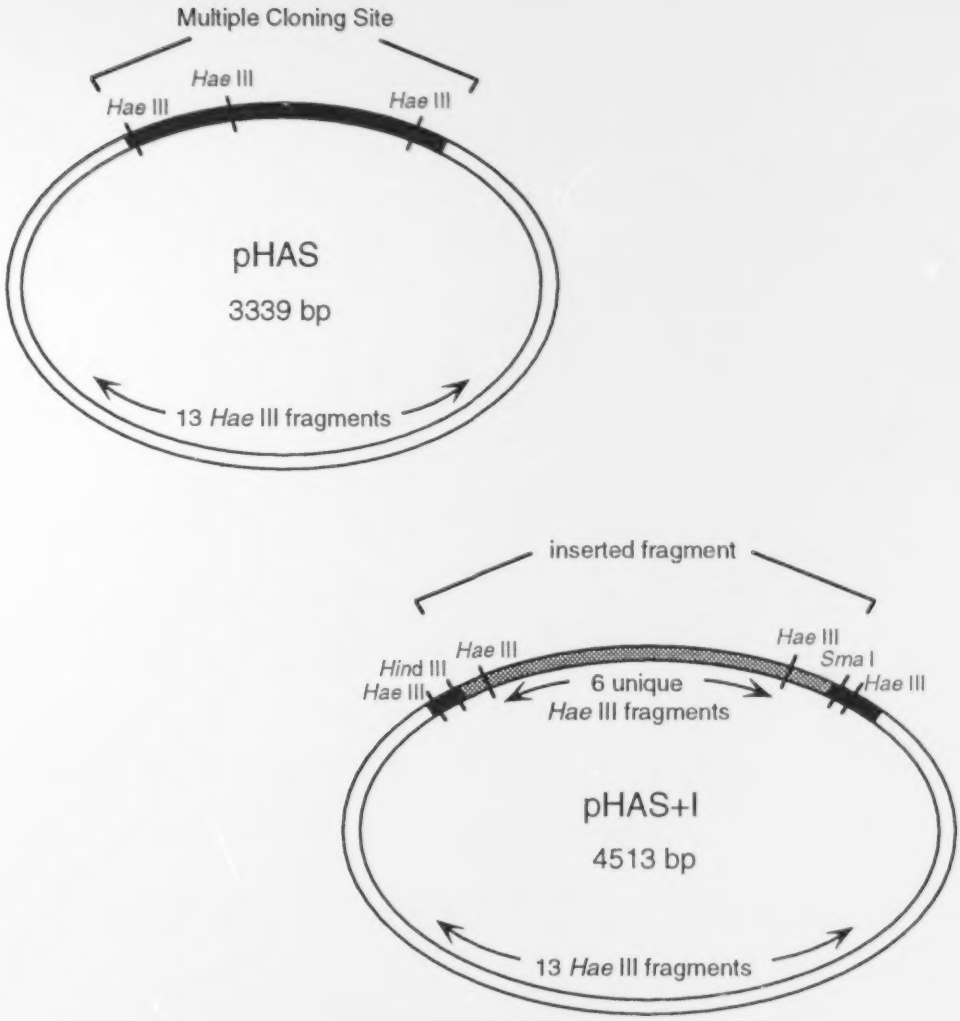


Figure 3. Diagramatic representation of the plasmids used in this study. On the pHAS plasmid, the black region identifies the multiple cloning site (MCS) and the white region represents the plasmid backbone. On the pHAS+I plasmid, the grey region indicates the inserted DNA fragment. The white backbone remains unchanged.

Life Technologies, Burlington, Ontario, and VWR Canlab, Edmonton, Alberta. Media was supplemented with ampicillin from Wyeth-Ayerst Canada Inc, Winnipeg, Manitoba.

Mycobacterial cells and Lowenstein-Jensen (LJ) slants were obtained from the mycobacterial lab of the Provincial Lab of Public Health, Edmonton, Alberta. *M tuberculosis* cells were grown on LJ slants at 37°C. The *M bovis* LJ slants were supplemented with 0.25% pyruvic acid and these organisms grew at 37°C in 5%CO_2 atmosphere. All cultures were maintained by Lillian Cook in the mycobacterial lab of the Provincial Lab in Edmonton.

4.2 Chemicals and reagents

The lipase, proteinase K and lysozyme enzymes required for the genomic DNA isolations were purchased from Sigma-Aldrich Canada Ltd (Mississauga, Ontario). For the biotin labeling and detection, the PHOTOPROBE^R biotin was purchased from Dimension Laboratories, Mississauga, Ontario, the Dynabeads^R M-280 streptavidin was supplied by Dynal Inc., Lake Success, New York, and the avidin-horseradish peroxidase conjugate came from Bio-Rad Laboratories, Richmond, California. Boehringer Mannheim, Laval, Quebec supplied the Klenow enzyme, the positively charged nylon membranes, and the Digoxigenin (DIG) DNA labeling and DIG detection kits.

Beta-agarase I enzyme, *Hae* III restriction endonuclease, T4 polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs, Mississauga, Ontario. The *Taq* DNA polymerase, the 2'-

deoxynucleoside 5'-triphosphates (dNTPs) and the molecular weight markers were obtained from Canadian Life Technologies, Burlington, Ontario. Amicon Canada, Oakville, Ontario supplied the Centricon^{R}-100 concentrators.

Other chemicals were from VWR Canlab, Edmonton, Alberta, Fisher Scientific, Edmonton, Alberta, Sigma Chemical Company, St. Louis, Missouri and Mandel Scientific Company Limited, Guelph, Ontario.

The Type 55 positive/negative polaroid film was purchased from Polaroid Canada Inc., Rexdale, Ontario, and the X-Omat AR autoradiograph film was from Kodak Canada, Inc., Ottawa, Ontario.

4.3 Purification of DNA fragments

Four common techniques were used in this study to purify the various DNA fragments. Unless the DNA was labeled, the yield and purity of the fragments were determined by spectrophotometric absorption at 260 and 280 nm. For double stranded DNA, an optical density reading at 260 nm (A_{260}) of 1.0 represented 50 μg/ml of DNA (Gallagher, 1994). DNA samples with an absorbance ratio of 1.7 (A_{260}/A_{280}) or higher were considered clean.

A Centricon^{R}-100 concentrator (Amicon Canada Inc, Oakville, Ontario) was used to purify DNA fragments in solution as follows. The volume was increased to 1 ml with sterile water. This was placed in the sample reservoir attached to the filtrate cup, and the device was spun at 530 X g for 10-15 minutes. The DNA sample was washed twice with 1 ml of sterile water, spinning for 10 - 15 minutes each time, and the final spin decreased the sample volume to

between 70 and 100 μl leaving the DNA fragments dissolved in water.

Gel slices containing DNA fragments were treated with β-agarase I enzyme according to the product insert. Digestion typically involved 200 μl of melted LMP or NuSieve^{R} agarose slice, 22 μl of 10X buffer (100 mM Bis-Tris-HCl, pH 6.5, 10 mM EDTA) and 1-2 U of enzyme at 40°C for at least 30 minutes. Rather than the final precipitation steps described by the manufacturer, the digestion reaction was filtered through a Centricon^{R}-100 concentrator as described above, leaving the final DNA sample dissolved in sterile water.

For the mycobacterial genomic DNA trapped in 0.75% (w/v) LMP agarose blocks, β-agarase I digestion involved 100 μl of melted slice, 11 μl of 10X buffer and 1 U of enzyme incubating at 40°C for at least 30 minutes. The tubes were spun at 12,000 X g for 3 minutes and the DNA containing supernatant was cleaned through a Centricon^{R}-100 concentrator as described above, except that the DNA was washed with TE buffer rather than sterile water.

DNA was precipitated from solution by addition of 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ice cold ethanol. The sample was vortexed well and incubated at -20°C for at least 1 hour. The DNA was pelleted by centrifugation at 12,000 X g for 30 minutes. The supernatant was removed and the pellet rinsed once with 1 ml of ice cold 70% ethanol, dried slightly and resuspended in sterile water.

The electroelution of fragments trapped in conventional agarose gel slices followed the procedure of Sambrook *et al* (1989). Gel slices were placed in dialysis tubing (3/4" prepared dialysis tubing, Canadian Life Technologies, Burlington, Ontario) with 2-3 ml of 0.5X TBE, enough to surround the slice.

Tubing was sealed and the DNA fragments were electrophoresed into the surrounding buffer at 70V for 45 minutes. Current was reversed for 1 minute and the buffer in the tubing was transferred to microcentrifuge tubes. Tubes were spun at 12,000 X g for 3 minutes and the DNA in the supernatant was further purified either through a Centricon^{R}-100 concentrator or by precipitation.

4.4 Plasmid DNA study

Figure 4 shows a summary of the steps used to identify the unique pHAS+I fragments.

4.4.1 Plasmid DNA isolation

Electroporation of the two plasmids followed the recommendations outlined by the manufacturer of the Gene Pulser apparatus and Pulse controller (Bio-Rad Laboratories, Richmond, California). In summary, DH5α electroporation cells were removed from the -70°C freezer and placed on ice to thaw. A 60 μl aliquot was transferred to a cold microcentrifuge tube containing 0.5 μg of plasmid DNA. The solution was gently mixed, placed into a chilled electroporation cuvette (0.2 cm gap, Bio-Rad), and pulsed at 2.5 kV, 200 ohms and 25 μFD. Immediately following the pulse, 0.5 ml of SOC media was added to the cuvette and the mixture was transferred to a room temperature polypropylene tube. A second 0.5 ml aliquot of SOC media was used to rinse the cuvette and added to the original sample. The mixture was incubated at

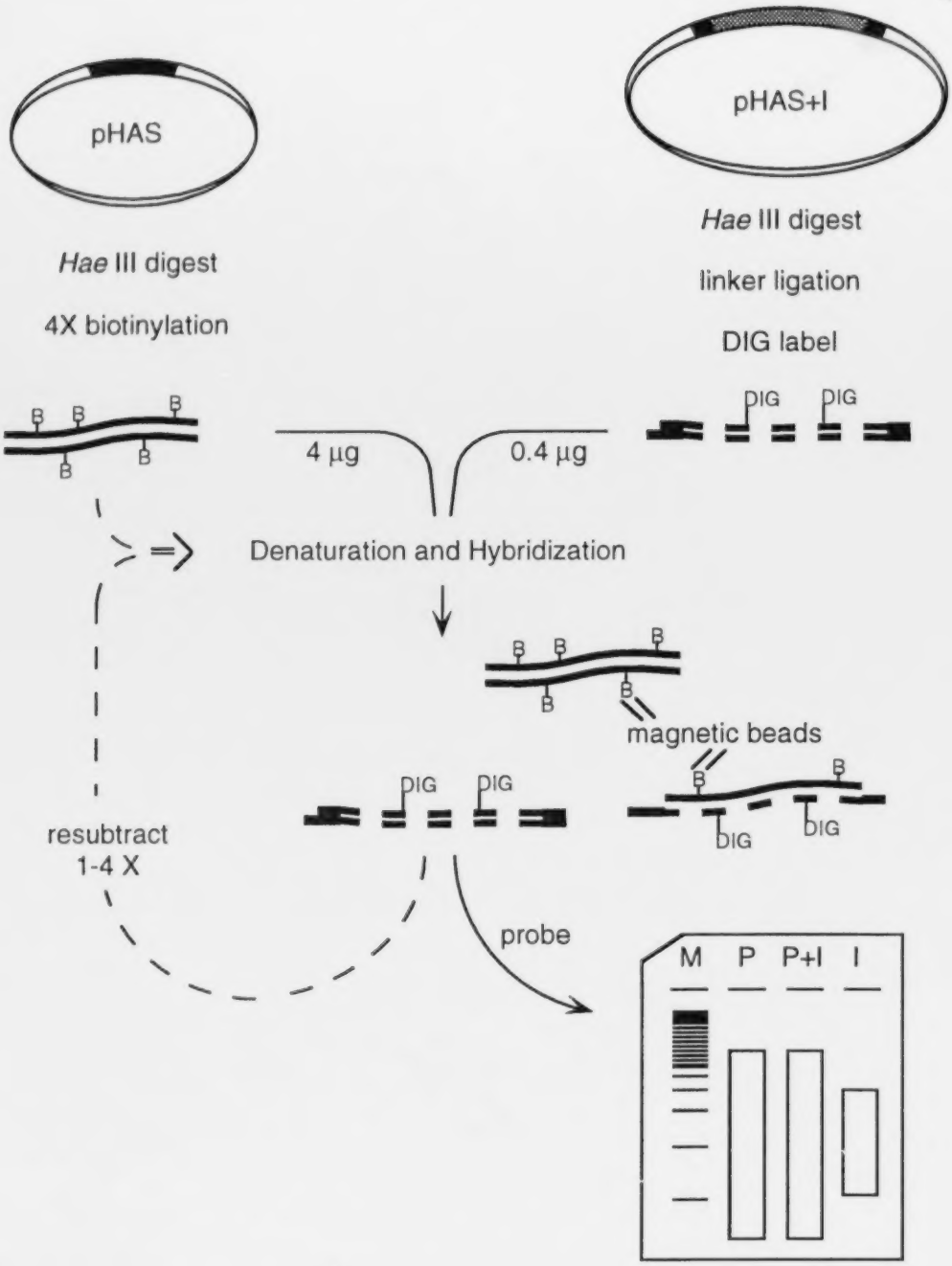


Figure 4. Diagramatic representation of the subtraction scheme used in the plasmid DNA studies. DIG: Digoxigenin label; B: biotin label; M: 100 bp molecular weight marker; P: pHAS plasmid fragments; P+I: pHAS+insert fragments; I: insert fragments.

37°C for 1 hour with shaking (240 rpms) and then 5, 50, 100 and 200 μl aliquots were plated onto LB agar plates containing 100 μg/ml ampicillin, 100 μg/ml X-gal and 240 μg/ml IPTG. Plates were incubated at 37°C for 17 hours.

For each plasmid, one colony of transformed cells was used to inoculate 200 ml of Superbroth containing 100 μg/ml ampicillin. The cultures were incubated at 37°C for 25 hours with agitation at 220 rpm and then placed at 4°C.

Plasmid DNA was isolated using the alkali lysis method described by Heilig *et al* (1994) with modifications. Overnight cultures were centrifuged at 4,200 X g for 15 minutes at 4°C. The supernatant was removed and the pellet was completely resuspended in 5 ml of lysis buffer (50 mM glucose, 25 mM Tris, pH 8.0, 10 mM EDTA, 5 mg/ml lysozyme). The mixture sat on ice for 10 minutes. Ten ml of freshly prepared 0.2N NaOH, 1% SDS was added and mixed in by inverting the tube several times. The tube sat at room temperature for 5 minutes and then received 7.5 ml of ice cold 7.5 M ammonium acetate, pH 7.5. The solution was mixed by inverting several times and sat on ice 5 minutes. Debris from the mixture was removed by pelleting at 12,000 X g for 10 minutes at 4°C. The clear supernatant was transferred to clean tubes in 10 ml aliquots and DNase-free RNase A was added to a final concentration of 25 μg/ml. The solutions were mixed well by vortexing and incubated at 37°C for at least 1 hour. Two volumes of phenol/chloroform (50% phenol, buffered with 1 M Tris, pH 8.0 and 50% chloroform, prepared as a combination of chloroform and isoamyl alcohol in a 24:1 ratio) was added and the solutions vortexed for 30 seconds. Samples were centrifuged at 12,000 X g for 10 minutes. The top aqueous phases were transferred to clean tubes containing an equal volume of ice cold

isopropanol. The solutions were mixed well and sat at -20°C overnight.

Plasmid DNA was pelleted by centrifugation at 12,000 X g for 30 minutes at 4°C. The supernatants were removed and the pellets rinsed twice with a few ml of ice cold 70% ethanol. The pellets were allowed to dry slightly, and each was resuspended in 750 μl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The solutions were all transferred to one clean tube and the original tubes rinsed with TE buffer to make a total final volume of 3 ml. Nine ml of CsCl stock (1.2 gm/ml water [w/v], filtered through 0.45 filter), was mixed in and the solution was transferred to a Ti 70 ultracentrifuge tube (38 ml volume). After adding 300 μl of ethidium bromide stock (10 mg/ml), the tube was filled to the sub-neck level with CsCl:water solution (4:1 of CsCl stock:sterile water). Tubes were balanced to within 0.013 gm, heat sealed and spun at 311,000 X g for 20 hours at 19°C. Following centrifugation, the lower plasmid band was removed using a 16G needle and a 5 ml syringe. Ethidium bromide was extracted away by adding an equal volume of salt saturated isopropanol, mixing and letting the layers separate at room temperature for 3-5 minutes. The top layer was discarded and the extraction repeated 3 more times, until all visible ethidium bromide colour was gone. TE buffer was added to a point where the total volume was 4-5 times the original volume of the DNA band. The solution was aliquoted into clean tubes containing an equal volume of ice cold isopropanol, the solutions were vortexed well and allowed to sit at -20°C overnight. The DNA was pelleted by spinning at 12,000 X g for 30 minutes at 4°C. The pellets were rinsed 3 times with 1 ml of ice cold 70% ethanol, resuspended and combined in a total volume of 400 μl TE buffer. The plasmid DNA was precipitated once again by adding 40

µl of 3 M sodium acetate and 1 ml of ice cold ethanol. The sample sat at -20°C for at least 30 minutes and spun in a microcentrifuge at 12,000 X g for 10 minutes at 4°C. Pellet was rinsed twice with 1 ml of ice cold 70% ethanol, allowed to air dry a few minutes and resuspended in 400 µl of TE. Yield and purity of DNA was determined by measuring the absorbance at wavelengths of 260 and 280 nm in an Ultraspec^{R} Plus spectrophotometer (Pharmacia LKB Biochem Ltd, Cambridge, England).

4.4.2 Restriction enzyme digestion

Insert DNA was isolated by digesting the pHAS+I plasmid with *Hind* III and *Sma* I at a concentration of 1.5 units (U) enzyme per µg of DNA at 37°C for 3.5 hours. The two digested products were electrophoresed through a 1% (w/v) low melting point (LMP) agarose gel in 1X TBE electrophoresis buffer (89 mM Tris-base, 89 mM Boric Acid, 20 mM EDTA) containing 0.5 µg/ml ethidium bromide. A gel section containing the insert DNA was excised and the DNA released using β-agarase I as described in section 4.3.

Digestions with the *Hae* III restriction endonuclease were as follows. In a final volume of 50-55 µl, 20 µg of each plasmid was combined with 40 U of enzyme and the appropriate buffer. The insert DNA reaction involved 30 µg of DNA with 35 U of enzyme and the appropriate buffer. The mixtures were incubated at 37°C for at least 1.5 hours. Digestion was verified by electrophoresis through a 2% (w/v) agarose gel in 1X TBE buffer containing 0.5 µg/ml ethidium bromide and exposure of the gel to ultraviolet (UV) light. Gels

were photographed with a Polaroid MP-4 Land camera using Type 55 positive/negative Polaroid film.

4.4.3 Preparation of biotinylated pHAS fragments

Biotin labeling of the pHAS fragments followed the Vector Laboratories (Burlingame, California) PHOTOPROBE^R Biotin Product Information sheet, with modifications. For the first reaction, 50 μg of Centricon^R-100 purified DNA fragments were in 70 μl of sterile water. Under safe light conditions, an equal volume of PHOTOPROBE^R Biotin stock solution (1μg/μl) was added to the DNA sample on ice. With the open tube surrounded by ice, the mixture was irradiated 10 cm below a sunlamp (mercury vapor bulb) for 15 minutes. An equal volume of 0.1 M Tris-HCl, pH 9.5 was added followed by one volume of 2-butanol equal to the total volume of DNA/Biotin/Tris-HCl mixture. The resulting solution was vortexed for 30 seconds and spun in a microcentrifuge for 3-4 minutes. The upper layer was discarded and a second volume of 2-butanol was added, equal to the amount used in the first extraction. After vortexing and spinning, the bottom aqueous phase was transferred to a clean tube. The biotinylated DNA was precipitated by addition of 1/5 volume of 1 M NaCl and 2.5 volumes of cold 95% ethanol. The sample was vortexed for 1 minute and sat at -20°C for at least 1 hour. The DNA was pelleted by centrifugation at 12,000 X g for 30 minutes. The pellet was rinsed once with 1 ml of ice cold 70% ethanol, dried slightly and resuspended in a volume of water making the DNA concentration 0.5 μg/μl, assuming all the original DNA was recovered. The biotinylation procedure was

repeated three more times for a total of four labeling reactions per DNA sample.

Biotinylated DNA fragments were attached to a positively charged nylon membrane using a slot blot manifold (Bio-Rad, Mississauga, Ontario) as follows. The membrane was prewetted with 6X SSC (0.9 M NaCl, 0.09 M sodium citrate) and placed in the apparatus on three Bio-Dot S.F. filters prewetted in 6X SSC. Under vacuum, the wells were rinsed with 6X SSC and the DNA sample (1 μg/slot) was suspended in 6X SSC, loaded into the wells and applied to the membrane. The membrane was carefully removed from the apparatus and allowed to dry 30 minutes at 37°C. The DNA was crosslinked to the membrane using a UV Crosslinker at 120,000 $\mu J/cm^2$ (Fisher Scientific, Edmonton, Alberta). Biotinylated DNA was detected using a procedure from Erica Pfeiffer (personal communication). The following protocol was used with 20 cm^2 membranes and volumes were adjusted for larger membranes. The membrane was sealed in a hybridization bag (Canadian Life Technologies, Burlington, Ontario) with 2 ml of 1% blocking reagent (Boehringer Mannheim, Laval, Quebec) in PBS and incubated for at least 2 hours at room temperature on a rocking platform. The membrane was rinsed for 5 minutes in 2X SSC with gentle agitation at room temperature and then placed in a second hybridization bag with 2 ml avidin-horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, California) diluted 1:400 in saturation buffer (50 mM Tris-HCl, pH 7.8, 0.1% Tween-20, 0.5% BSA, 0.05% sodium azide). The conjugate was allowed to bind to the biotinylated DNA during incubation at 37°C for 15 minutes on the rocking platform. The membrane was removed from the bag and washed in twelve, 50 ml changes of TBS (20 mM Tris-HCl, pH 7.6, 500 mM NaCl), 10 minutes per

wash. The blot was equilibrated in 50 ml of sodium acetate buffer (0.2 M acetic acid and 0.2 M sodium acetate combined to a pH of 5.0) for 5 minutes. During this time, fresh colour developer was made, mixing carefully to avoid precipitating reagents. In a disposable tube wrapped in foil, 2.5 ml N'N'-dimethylformamide was added to 10 mg 3-amino-9-ethylcarbazole. In a darkened room, just before use, the ethylcarbazole solution was added to 47.5 ml 200 mM sodium acetate buffer. Fifty μl 30% hydrogen peroxide was quickly mixed in and the entire solution poured onto the membrane. Colour was allowed to develop in the dark for 15-30 minutes, depending on intensity of the positive control. Development was terminated by rinsing the membrane in sterile distilled water for at least 1 hour. The membrane was allowed to dry on filter paper overnight and stored in the dark to maintain the colour intensity.

The procedure for capture of biotinylated DNA with streptavidin coated magnetic beads (Dynabeads^{R} M-280 Streptavidin) followed the manufacturer's instructions. First, the beads were resuspended in their original vial and, using sterile technique, an aliquot was removed to a sterile microcentrifuge tube. The tube was placed into a magnetic particle concentrator (MPC) for 1 minute and the supernatant discarded. The tube was removed from the concentrator and the beads were gently resuspended in PBS (pH 7.4), 0.1% BSA, the same volume as the original aliquot. The tube was placed back in the concentrator for 1 minute, the supernatant removed, and a second PBS/BSA washing was done. Following these washes, the beads were suspended in 2X binding buffer (10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl, 0.1% Tween-20, pH 7.5) and combined with an equal volume of biotinylated DNA sample (total volume of at least 150 μl).

Binding of the DNA to the beads proceeded at room temperature on a rocker platform for 30 minutes. The tube was then placed into the MPC for 1 minute and the supernatant carefully removed to a clean tube. For repeated binding, this supernatant was placed directly into a tube containing freshly washed beads and allowed to incubate again at room temperature for 30 minutes.

Success of biotinylation was evaluated as follows. Each time that DNA fragments were biotinylated, a small quantity of the sample was tested to determine the amount of magnetic beads and number of capture treatments required to remove all traces of biotin signal. In conjunction with this, an identical sample was analysed for the presence of unlabeled DNA fragments. Two μg of biotinylated DNA was subjected to the capture protocol and the resulting supernatant was divided into two aliquots. One sample was UV crosslinked to a positively charged nylon membrane (as described earlier) and subjected to the biotin detection protocol. The second sample was dotted onto a 1% (w/v) agarose gel containing 1 μg/ml ethidium bromide in 2 μl aliquots, allowing the droplet to soak into the agarose between applications. The gel incubated at room temperature for 20 minutes and was viewed under UV light.

4.4.4 Oligonucleotide linkers

Synthetic linkers were designed to be ligated to the *Hae* III digested DNA fragments. The two oligonucleotides were complementary, forming a double stranded linker with one blunt end and the other a 3' noncohesive overhang. Their sequences and orientation are shown in Figure 5. The oligonucleotides

Figure 5. Synthetic oligonucleotides used in this study.

were purchased from the ADRI DNA Synthesis Laboratory where they were synthesized using a PCR-mate 391 DNA synthesizer (Applied Biosystems, Foster City, California) according to the manufacturer's instructions. Following deprotection and desalting, each oligonucleotide was further purified through a C-18 Sep-pak^R cartridge (Millipore Corporation, Milford, Mass.) according to John Elliott (personal communication). The cartridges were charged with 10 ml pure acetonitrile and washed with 10 ml sterile distilled water. The oligonucleotide was dissolved in 10 ml crush and soak buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, pH 6.2) and loaded onto the cartridge by passing the solution through the Sep-pak^R 3 times. The cartridge was washed with 10 ml sterile distilled water and the oligo was eluted from the matrix using 1 ml aliquots of 20% acetonitrile. Each aliquot was collected separately, dried down, and the pellets dissolved in a total of 100 μl TE buffer. Yield and purity of DNA was determined from the spectrophotometric absorbance at 260 and 280 no. For short, single stranded oligonucleotides, a 1.0 A_{260} reading represented 25 μg/ml of DNA.

The CL-02 oligonucleotide was phosphorylated according to Tabor (1987). The 20 μl reaction mixture included 1X reaction buffer, 1 mM ATP, 20 units T4 polynucleotide kinase and 10 μg CL-02. The reaction proceeded at 37°C for 60 minutes followed by enzyme inactivation at 65°C for 20 minutes.

Equal molar amounts of each oligonucleotide were annealed by combining 9.5 μl of CL-01 (0.5 μg/μl) and 11.5 μl of CL-02 (0.5 μg/μl) at room temperature for at least 15 minutes producing a 0.5 μg/μl annealed linker sample.

The annealed oligonucleotide linkers were ligated to the pHAS+I *Hae* III fragments using the overnight ligation reaction as described by John Elliott (personal communication). Specifically, reaction volumes were 10.5 μl and included 2 μg pHAS+I fragments, 4 μg annealed linkers (10X molar excess) and 20U T4 DNA ligase in 1X ligase buffer (50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 50 μg/ml BSA). Reaction mixtures were incubated in a 14°C waterbath for at least 17 hours. To ensure ligation, two control reactions were run simultaneously with the test ligation reaction. The first was the test reaction without the ligase enzyme, and the second was the test ligation reaction without the linkers. Comparison of the three reactions was performed by running the products in a 2% (w/v) agarose gel in 1X TBE buffer.

Ligated DNA fragments (pHAS+I+L) were purified using the Centricon^R-100 concentrator. Spectrophotometric absorbance at 260 nm indicated the yield and the DNA was diluted to 0.5 μg/μl in sterile water.

4.4.5 Preparation of DIG labeled pHAS+I+L fragments

The pHAS+I+L fragments were labeled with Digoxigenin (DIG) according to the DIG labeling kit product insert, with modifications. The 20 μl labeling reaction included 1μg of Centricon^R-100 purified pHAS+I+L fragments (denatured in a boiling water bath for 10 minutes), 2 μl 10X dNTP mixture (vial 6 in kit which contains DIG-dUTP), 2 μl 10X buffer (2 mg/ml BSA, 0.5M Tris-HCl, pH 7.2, 0.1 M $MgCl_2$, 1 mM DTT), 1.5 μg CL-01 oligonucleotide primer and 2U Klenow enzyme. The remainder of the 20 μl volume was sterile water and the

reaction was incubated at 37°C for at least 20 hours. The DNA fragments were purified using the Centricon^{R}-100 concentrators and stored in sterile water at 4°C.

4.4.6 Subtractive hybridization

For the first round of subtraction, biotinylated fragments were combined with the DIG labeled fragments in a 10:1 ratio. Specifically, 4 μg of biotinylated pHAS DNA in water was added to 0.4 μg DIG labeled pHAS+I+L fragments in water in a microcentrifuge tube on ice. One volume of 1 M sodium phosphate buffer (pH 6.8) was added. The mixture was covered with 50 - 100 μl of sterile mineral oil and placed in a heat block (Canlab, Edmonton, Alberta) containing water at 96-98^{0}C for at least 10 minutes. Following this denaturation step, the mixture was placed on ice and immediately transferred to a 65^{0}C waterbath where it incubated for at least 18 hours. Biotinylated DNA was removed from the solution using streptavidin coated magnetic beads as described earlier (section 4.4.3). For plasmid DNA subtractions, the mixture was removed from under the oil layer and two streptavidin absorption cycles were performed.

For subsequent subtractions, the supernatant remaining after the capture of the biotinylated fragments was purified through the Centricon^{R}-100 concentrator into sterile water, combined with 4 μg biotinylated fragments and another cycle of denaturing and overnight hybridization was performed.

4.4.7 Southern hybridization

Southern transfers were done according to Sambrook *et al* (1989). DNA fragments were electrophoresed for 3 hours at 6 V/cm through a 3% (w/v) NuSieve^R GTG^R agarose gel in 1X TAE electrophoresis buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.4). For the plasmid experiments, gel lanes contained 5 μg of each of the *Hae* III digested plasmids and 2 μg of *Hae* III digested insert DNA. The gel was washed in three changes of denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 15 minutes each, one 15 minute rinse in sterile water and three 15 minute washes in neutralizing solution (1 M Tris-HCl pH 7.4, 1.5 M NaCl). The overnight capillary transfer was set up in a large glass dish, with two strips of filter paper acting as a wick up onto a plastic support. The 10X SSC transfer buffer (1.5 M NaCl, 0.15 M sodium citrate) filled the dish and soaked the filter paper. The inverted gel was placed on the filter paper, and the edges were surrounded by plastic wrap. The positively charged nylon membrane (Boehringer Mannheim, Laval, Quebec) was floated on distilled water, prewetted in 10X SSC for at least 5 minutes and placed on top of the gel. The membrane was covered with three pieces of filter paper which had been soaked in 2X SSC and a thick stack of paper towels. A 500 gm weight was applied to the paper towels and the DNA was left to transfer for at least 20 hours. The paper towels and filters were removed and the well positions were marked on the membrane. The membrane was rinsed in 6X SSC for 5 minutes and then allowed to dry for 30 minutes at 37^{0}C. The DNA was crosslinked to the membrane by irradiation with a UV crosslinker (Fisher Scientific, Edmonton, Alberta) at 120,000 μJ/cm^2.

When not in use, the membranes were sealed in hybridization bags and stored at -20^{0}C.

The amount of probe used for each hybridization was based on 100 ng of presubtraction DNA. Specifically, 100 ng of DIG labeled pHAS+I+L was used in the first hybridization to provide the control signal before subtraction. Since the first subtraction reaction involved 400 ng of DIG labeled pHAS+I+L, one quarter of the product was saved and used as a probe for the second hybridization. Following the second subtraction, one third of the product was saved for use as the probe in the third hybridization. One half of the third subtraction product became the probe in the fourth hybridization and all of the fourth subtraction product was used as a probe in the fifth hybridization.

Southern membranes were incubated at 68^{0}C for at least one hour in prehybridization buffer (5X SSC, 1% blocking reagent from DIG detection kit, 0.1% N-lauryl sarcosine, 0.02% SDS). Initially, probes were denatured in a microcentrifuge tube at >96^{0}C in a boiling water bath for at least 10 minutes. After prehybridization, the solution was removed from around the membrane and fresh 68^{0}C prehybridization buffer was added and the bag resealed. On ice, the freshly denatured probe was diluted to 500 μl with a small amount of prehybridization buffer and added to the fresh solution surrounding the membrane using a 26G needle and 1 ml syringe. Once sealed with the denatured probe, the membrane was incubated at 68^{0}C for at least 6 hours, usually 15-18 hours overnight. The hybridization buffer was removed and stored at -20^{0}C for future use, and the membrane went through the DIG detection protocol. When the probes were used a second time, the entire hybridization

mixture (prehybridization buffer and probe) was placed in a boiling waterbath for at least 15 minutes.

Following detection, the membranes were stripped for reuse according to the detection kit information sheets. Membranes were rinsed in sterile water for 15 minutes with gentle agitation at room temperature followed by two 15 minute washes at 37°C in 0.2 N NaOH, 0.1% SDS and one final 10 minute rinse in 2X SSC at room temperature. Damp membranes were sealed in hybridization bags and stored at -20°C.

4.4.8 DIG detection

Membrane-bound, DIG labeled DNA fragments were detected according to the product insert. The following steps are for 35 cm^2 southern membranes and the volumes were increased proportionately for larger membranes. The membrane went through two stringency wash cycles with gentle agitation. The first cycle consisted of two washes at room temperature in 200 ml of 2X SSC, 0.1% SDS for 5 minutes each, and the second cycle was two washes at 68°C in 200 ml of 0.1X SSC, 0.1% SDS for 15 minutes each. The membranes was then incubated for 30 minutes in 80 ml of buffer II (1% blocking reagent in buffer I (0.1 M maleic acid, 0.15 M NaCl) pH 7.5) on a rocking platform at room temperature. Following this blocking step, the anti-DIG-AP conjugate was diluted 1:10,000 in 20 ml of buffer II and allowed to bind to the DIG label at room temperature for 30 minutes on the rocker. The membrane was washed twice for 15 minutes each time on the rocking platform in washing buffer (0.3% Tween-20 in buffer I).

Equilibration was in buffer III (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5) for 5 minutes. Lumigen PPD was diluted 1:100 in buffer III and was used to flood the membrane, which had been placed on plastic wrap on the counter. This was left for 10 minutes, with the occasional addition of more buffer to keep the membrane covered. The lumigen PPD solution was stored at 4°C and reused several times. The membrane was removed from under the lumigen solution, placed on filter paper and allowed to air dry for a few minutes (not to complete dryness). The damp membrane was sealed in a hybridization bag and sunk in a 37°C waterbath for 15 minutes. The bag was removed from the waterbath, dried well with paper towel and exposed to X-Omat AR autoradiograph film, usually for 30 minutes, depending on the strength of the signal. Film was developed by soaking in V307 X-ray developer-replenisher solution for 4 minutes, rinsed in water for 1 minute, soaked in V306 X-ray fixer solution for 4-6 minutes and rinsed in water for at least 1 hour (both developer and fixative were purchased from Christie Group Ltd, Toronto, Ontario).

4.5 Mycobacterial DNA study

Figure 6 shows a summary of the steps taken to identify unique *M bovis* DNA fragments. There are two steps which differ from the plasmid scheme (Figure 4). First, partial digests were used to produce differing sizes of *M tuberculosis* and *M bovis* fragments. Second, labeling of the *M bovis* target DNA occurred after the subtraction step, during PCR amplification.

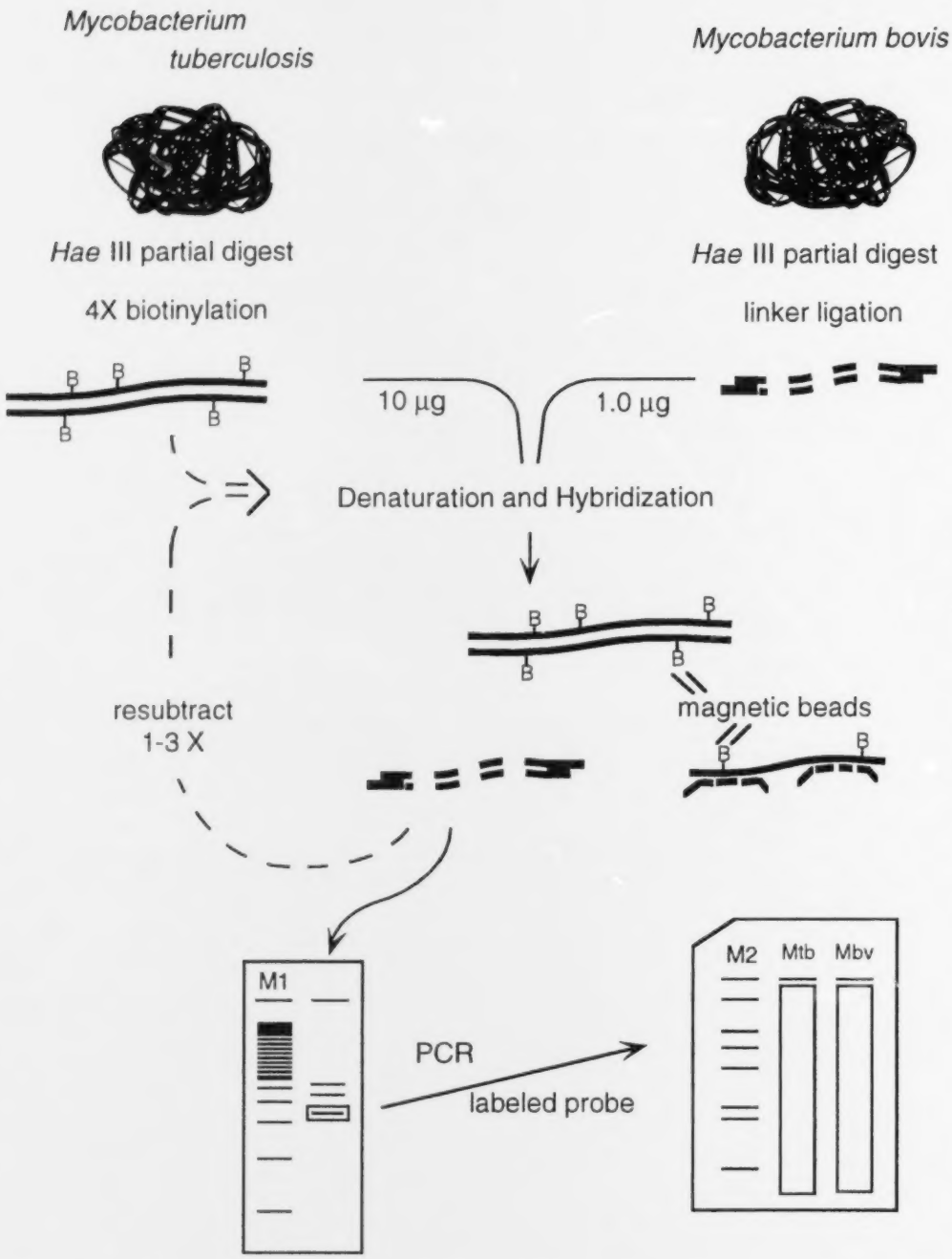


Figure 6. Diagramatic representation of the subtraction scheme used in the mycobacterial DNA studies. B: biotin label; M1: 100 bp molecular weight marker; M2: Lambda DNA-*Hind* III molecular weight marker; Mtb: *Eco*R 1 digested *Mycobacterium tuberculosis*; Mbv: *Eco*R 1 digested *Mycobacterium bovis*

4.5.1 DNA isolation

The isolation procedure used in this study was adapted from a procedure used in the Public Health Laboratory (Edmonton, Alberta). LJ slants were inoculated with 2 drops of a McFarland #1 standard suspension of each strain. *M tuberculosis* cells were incubated for 2-3 weeks at 37°C, and the *M bovis* cells were incubated for at least 6 weeks at 37°C in a 5% CO_2 environment on LJ slants containing 0.25% pyruvic acid. Once a confluent layer of growth was seen, cells were scraped from the LJ slants and transferred into 2 ml microcentrifuge tubes at a concentration of 200-300 mg cells (wet weight) per tube. The cells were suspended in 1 ml of TEN (50 mM Tris-HCl, pH 8.0, 100 mM EDTA, 150 mM NaCl) and heat inactivated in a 80-85°C waterbath for at least 1 hour. Complete sterilization was verified by inoculating two drops of this suspension to LJ slants and these were incubated at 37°C for a minimum of 6 weeks. The *M bovis* slants contained 0.25% pyruvic acid in 5% CO_2. No growth was seen.

The heat inactivated cell suspension was spun at 9,000 X g for 3 minutes. The cells were washed once in 1 ml TEN and then suspended in a solution of 20,000 U lipase in 1 ml of TEN. The suspension was incubated at 37°C for at least 2 hours. Equal volumes of the suspension and LMP agarose solution (1.5% agarose dissolved in 0.25 M EDTA, pH 8.0) were mixed, dispensed into 2X5X10 mm molds and cooled at 4°C for 15 minutes. Embedding the cells in agarose blocks protected the DNA from shearing while the thick mycolic acid wall and cell membrane were enzyme digested. This step also reduced the

possibility of aerosol production as the samples were manipulated, thus minimizing the risk to personnel.

The cold blocks were pushed from the molds into 1.5 ml microcentrifuge tubes containing digestion buffer I (6 mM Tris-HCl, pH 7.6, 1 M NaCl, 0.5% Brij-58, 100 mM EDTA, pH 8.0, 0.2% deoxycholate and 0.5% N-lauryl sacosine, with freshly added 20 μg/ml DNase-free RNase A and 2 mg/ml lysozyme). For each block, 0.3 ml of buffer was used and the blocks were incubated in a 37°C waterbath for 24 hours.

The blocks were transferred into 15 ml polypropylene tubes containing digestion buffer II (0.5 M EDTA, pH 8.0, 1% N-lauryl sarcosine with freshly added 1 mg/ml proteinase K) at 2 ml per block. Blocks were incubated in this buffer for 48 hours in a 50°C waterbath. Following incubation, these tubes were transferred to 4°C and stored for up to 5 months.

To purify the DNA, the blocks were washed twice at room temperature for one hour each time in digestion buffer III (TE, pH 7.5, with freshly added 1 mM phenylmethylsulfonyl fluoride). The blocks were further washed in four changes of TE, pH 7.5, at 1 hour per wash. Blocks were combined and heated at 70°C for 10 minutes and then 100 μl aliquots were transferred into microfuge tubes containing 11 μl of β-agarase I buffer (10X concentration) at 40°C. One unit of β-agarase I enzyme was added and the mixture incubated at 40°C for at least 30 minutes. The tubes were spun at 12,000 X g for 3 minutes and the DNA containing supernatant was further cleaned through a Centricon^{R}-100 concentrator (section 4.3) and stored in TE buffer at 4°C. Yield and purity of DNA was determined by spectrophotometric absorbance at 260 and 280 nm.

4.5.2 Preparation of DNA fragments

Since these species may be as high as 95 to 100% homologous (Imaeda, 1985; Grosskinsky *et al* 1989; Kusunoki *et al* 1991), unique segments were expected to be small and scarce. To be useful as a diagnostic marker, it was decided that the identified segment should be no shorter than about 300 bp. Because of the high homology, a unique segment may not be larger than about 800 bp. Thus, the *M bovis* genomic DNA was partially digested and the 300 to 800 bp fragments were purified. To allow for increased subtraction efficiency and minimize the time required to purify excessive amounts of DNA, 800 to 3,000 bp fragments of partially digested *M tuberculosis* H_{37}Ra DNA were purified.

For all partial digests, the *Hae* III restriction endonuclease was diluted to 1 U/μl in enzyme diluent (50 mM KCl, 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 1 mM DTT, 200 μg/ml BSA, 50% glycerol), stored at -20°C for 14 hours before using, and discarded after 24 hours. Genomic DNA was diluted to 0.1 μg/μl based on spectrophotometric readings at A_{260} and stored at 4°C. Typical digestions were 20 μg of diluted DNA incubating in *Hae* III 1X buffer with 3 U of the diluted *Hae* III enzyme. For the *M tuberculosis* DNA, digestion proceeded at 37°C for 20-35 minutes, depending on the activity of the specific enzyme dilution. For the *M bovis* DNA, digestion was at 37°C for 70-100 minutes depending on enzyme activity. Digestion products were immediately electrophoresed on a 0.8% (w/v) LMP agarose gel in 1X TAE usually at 7.5 V/cm for between 1.5 and 2 hours. Gel slices containing the appropriate DNA

fragments were cut out and stored at 4°C overnight.

DNA fragments were purified from the LMP gel slices by either β-agarase I digestion or electroelution into 0.5 X TBE (as described in section 4.3). In both cases, the DNA was finally purified and concentrated using a Centricon^{R}-100 filter. The fragments were stored in sterile water at 4°C.

Biotinylation of the *M tuberculosis* fragments was similar to the labeling performed on the pHAS plasmid fragments (section 4.4.3). Two microcentrifuge tubes each contained 55 μg of the 800-3,000 bp fragments in 110 μl were labeled with 4 cycles of the biotinylation reaction. The final pellets were resuspended and combined in a total of 220 μl of water making the DNA concentration 0.5 μg/μl, as in the plasmid study. One 5 μg aliquot of the labeled DNA was tested to determine the amount of magnetic beads required to completely remove the biotinylated DNA from the solution.

Linker ligation of the *M bovis* fragments was similar to the linker ligation for the plasmid work (section 4.4.4). A total of 4 ligation reactions were set up, each involving 2 μg of DNA fragments and 4 μg of annealed oligos (100X molar excess). The remaining reaction components were identical to the plasmid ligation reactions. The ligation products were combined and run on a 0.8% LMP gel in 1X TAE. The gel block containing the 300 to 900 bp fragments was excised and the DNA purified by electroelution and Centricon^{R}-100 cleaning into sterile water. Yield and purity of fragments was calculated from spectrophotometric absorbance at 260 and 280 nm.

4.5.3 Subtractive hybridization

The subtractive hybridization procedure and capture of biotinylated fragments was identical to the steps followed in the plasmid work (section 4.4.6), with these two exceptions. First, the hybridization involved 10 μg of *M tuberculosis* fragments and 1 μg of M *bovis* fragments in 0.5 M sodium phosphate buffer (pH 6.8). Second, following the hybridization, the reaction was split into two tubes and each aliquot was treated five times with 0.85 mg of washed magnetic beads.

4.5.4 PCR of subtraction products

To prepare the presubtraction control probe, PCR was used to amplify the fragments of *M bovis* DNA to which the linkers had successfully ligated. Reactions were performed in a 100 μl volume containing 10 ng of DNA fragments, 1X PCR buffer (20 mM Tris-HCl, pH 8.3, 50 mM KCl, 1 mM $MgCl_2$), 0.2 mM of each 2'-deoxynucleoside 5'-triphosphate (dNTP), 2 μM CL-01 oligonucleotide and 2.5 U of *Taq* DNA polymerase. Amplification was carried out in a TwinBlock™ System EasyCycler™ Series temperature cycler (Ericomp Inc., San Diego, California). Reaction cycles were 10 minutes at 94°C followed by 35 repeated cycles of 1 minute denaturing at 94°C, 1 minute annealing at 50°C and 2 minutes extension at 72°C. A final incubation was at 72°C for 10 minutes. Reactions were often left to run overnight and the samples would cool to room temperature when the cycler was finished.

PCR was used to amplify the fragments of *M bovis* remaining after each subtractive hybridization cycle. The amount of template DNA included in the reaction was based on 100 ng of presubtraction DNA. Specifically, after the first subtraction started with 1 μg of *M bovis* DNA fragments, one tenth of the volume of the subtracted DNA product was removed and included in the first PCR. After the second subtraction, one ninth of the volume was removed for amplification via PCR and likewise, after three subtractions, one eighth of the volume was used as the template DNA sample. The reaction conditions were the same as described above for the presubtraction control.

For analysis of the PCR products, 10 μl of the reaction sample was mixed with 2 μl of 6X loading buffer and electrophoresed in a 2% (w/v) agarose gel made in 1X TBE and containing 0.5 μg/ml of ethidium bromide.

In cases where the PCR products were faint or not visible in the 2% (w/v) agarose gel, 1 μl of the reaction product was used as template DNA in a second reaction with conditions and cycling times as described above. This was required to analyse the low amounts of product remaining after the third subtraction procedure.

4.5.5 DIG labeling via PCR

Labeling reactions were performed in 100 μl volumes containing 1X PCR buffer (20 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.25 mM $MgCl_2$) , 1X DIG dNTP mixture (0.1 mM dATP, 0.1 mM dGTP, 0.1 mM dCTP, 65 μM dTTP, 35 μM DIG-dUTP), 2 μM CL-01 oligonucleotide and 2.5 U of *Taq* DNA polymerase.

Amplification was carried out in the Ericomp TwinBlock™ System EasyCycler™ Series temperature cycler. Reaction cycles were 10 minutes at 94°C followed by 25 repeated cycles of 1 minute denaturing at 94°C, 1 minute annealing at 50°C and 2 minutes extension at 72°C. A final incubation was at 72°C for 10 minutes. Reactions were often left to run overnight and the samples would cool to room temperature when the cycler was finished. Labeling was verified by comparing the migration of labeled versus unlabeled fragments through a 2% (w/v) agarose gel in 1X TBE buffer. The PCR products were electrophoresed through a 3%(w/v) NuSieve^R gel in 1X TBE and the labeled fragments were excised.

4.5.6 Purification of individual fragments

Twenty μl of the second PCR product resulting from the third subtraction procedure was electrophoresed through a 1% (w/v) LMP agarose gel containing 0.5 μg/ml ethidium bromide in 1X TBE buffer. In order to analyse all visible subtraction products, four sections of the gel lane were excised as illustrated in Figure 7. Block #1 contained fragments from 800 to 480 bp, block #2 contained the 480 to 370 bp fragments, block #3 contained those from 370 to 300 bp and the final block (#4) ranged from 300 to 270 bp.

Block #1 was heated to 68°C and one fifth of the volume was included in the first DIG labeling PCR with conditions as described in section 4.5.5. Forty μl of the reaction products from this first block were electrophoresed through a 3% (w/v) NuSieve^R gel in 1X TBE and fragments 1A and 1B were individually excised. One fifth of each slice was used as template DNA in a second DIG

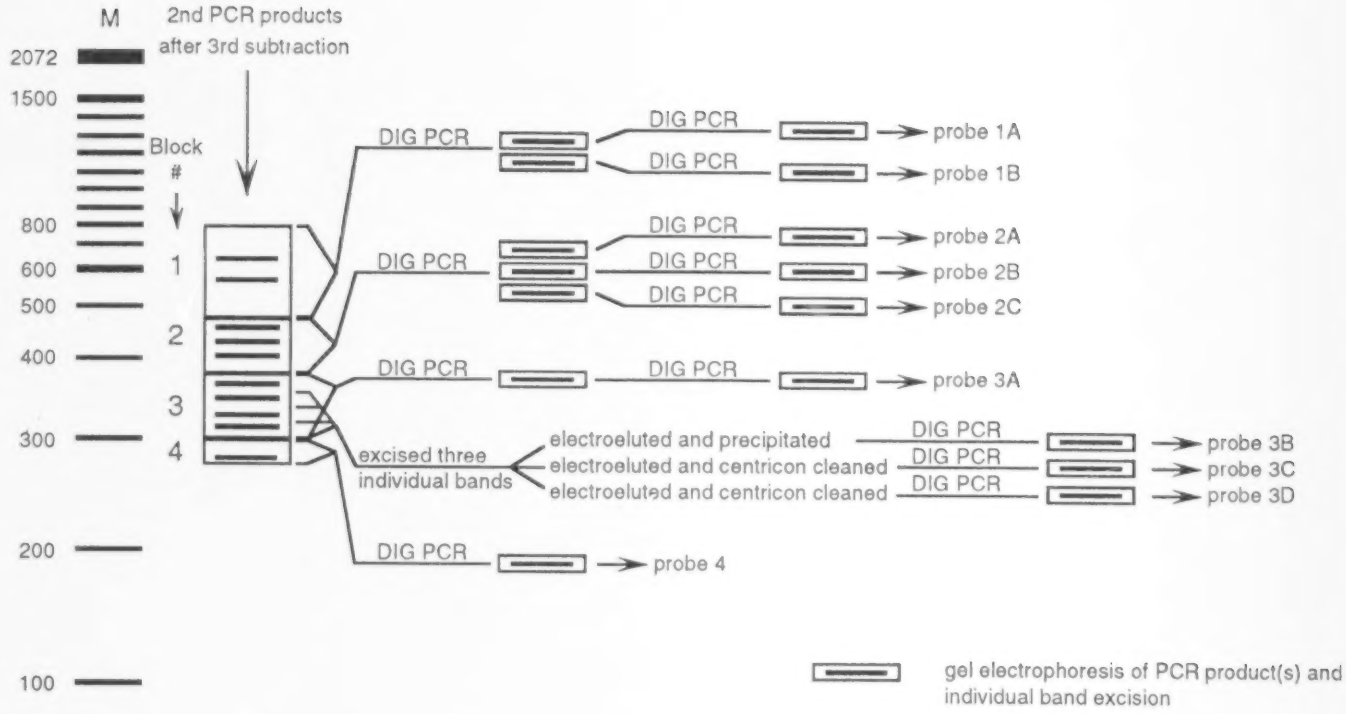


Figure 7. Diagramatic representation of the fragment purification steps.

labeling PCR. To gel purify the probes, the two reaction products were electrophoresed through a 3% (w/v) NuSieve^R gel and the individual bands were excised and stored at 4°C as probes 1A and 1B.

Block #2 was heated to 68°C and one tenth of the volume was included in the first DIG labeling PCR. The reaction products were electrophoresed through a 3% (w/v) NuSieve^R gel in 1X TBE and fragments 2A, 2B and 2C were individually excised. One tenth of slices 2A and 2B and one fifth of slice 2C provided template DNA for the second DIG labeling PCRs. The three separate PCR products were gel purified as described for block #1 and the individual bands were stored at 4°C as probes 2A, 2B and 2C.

Block #3 was heated to 68°C, and one tenth of the volume was template DNA for the first DIG labeling PCR and the products were gel purified. Fragment 3A was excised and one fifth of the slice volume was included in the second DIG PCR. The reaction products were gel purified and fragment 3A was excised and stored at 4°C. From block #3, three remaining fragments were purified as follows: 60 μl of the second PCR product following the third subtraction was electrophoresed through a 3% (w/v) NuSieve^R gel in 1X TBE and fragments 3B, 3C and 3D were individually excised. The DNA was electroeluted from each slice and purified via precipitation or through a Centricon^R-100 concentrator (section 4.3). The yield was determined spectrophotometrically and 400 ng was electrophoresed through a 3% (w/v) NuSieve^R gel in 1X TBE. The three fragments of interest were excised and became template DNA in separate DIG labeling PCRs. The PCR products were gel purified and the individual bands were stored at 4°C as probes 3B, 3C and

3D respectively.

Block #4 provided the final probe. The first DIG PCR produced only one visible DNA product, and this fragment was gel purified and stored at 4°C as probe 4.

4.5.7 Southern hybridizations

One membrane was prepared following the Southern transfer technique described earlier in the plasmid studies. The modifications were the source of DNA fragments and the type of gel. Mycobacterial DNA was digested as follows. Five μg of each of the mycobacterial genomic DNA samples was digested using 25U of the *Eco*R I restriction endonuclease enzyme. For each reaction, the DNA and enzyme were combined in 1X React 3 buffer and incubated at 37°C for 40 hours. The two samples were loaded into the wells of a 0.6% (w/v) agarose gel and electrophoresed at 0.4 V/cm using 1X TBE buffer containing 0.5 μg/ml ethidium bromide. The gel was washed in denaturing and neutralizing solutions and set up for the Southern transfer exactly as described in the plasmid work (section 4.4.7). The resulting membrane was stored at -20°C, sealed in a hybridization bag.

The control probe was prepared via PCR amplification of 10 ng of *M bovis* target DNA using the CL-01 oligonucleotide as the primer which bound to its complementary linker segment at the ends of the target DNA and primed the labeling reaction. Reaction conditions were exactly as described in section 4.5.5. The PCR products were Centricon^{R}-100 purified (section 4.3) into 100 μl

of sterile water to remove the excess primer, enzyme and dNTPs. For hybridization, 5 µl of control probe solution was denatured and included in the overnight reaction.

Each of the ten DIG labeled probes were used to detect complementary sequences on the Southern membrane. The amount of probe used for each hybridization varied from one half to nine tenths of the probe volume, based on the intensity of the labeled fragment when visualized under UV illumination during the final excision step. The hybridization conditions and detection steps were exactly as described for the plasmid hybridizations and detection (section 4.4.7 and 4.4.8).

Following each detection, the DIG labeled probe was stripped from the membrane exactly as done for the plasmid work (section 4.4.7). When not being used, the damp membrane was stored at -20°C in a sealed hybridization bag .

5.0 RESULTS AND DISCUSSION

5.1 Plasmid DNA study

A model system based on two slightly different plasmid populations was designed to test and validate the fragment preparation and subtractive hybridization steps. The driver DNA population was the 3339 bp pHAS plasmid shown in Figure 3. As described by Smith *et al* (1992), this plasmid is a modified version of the pBluescript KS- vector (Stratagene, La Jolla, California). The target DNA population was the same pHAS plasmid containing a 1543 bp insert (pHAS+I in Figure 3) and the insert represents DNA that is unique to this target population. To be successful, the model system must purify the insert sequences from a solution containing DNA fragments from both populations.

5.1.1 Restriction enzyme digests

Figure 8 compares the fragments generated by digestion of the plasmid samples with the *Hae* III restriction endonuclease. The digest profiles correspond to the expected profiles calculated from the DNA sequence (John Elliott, personal communication). Digestion of the pHAS plasmid (lane 2) produced 15 individual fragments: 767, 458, 434, 294, 267, 243, 174, 170, 142, 125, 102, 80, 54, 18 and 11 bp. Fragments above the 100 bp size could be consistently seen on a 3% (w/v) NuSieve^{R} gel. Digestion of the pHAS+I plasmid (lane 3) resulted in 21 fragments: 767, 458, 446, 434, 309, 267, 262, 243, 174,

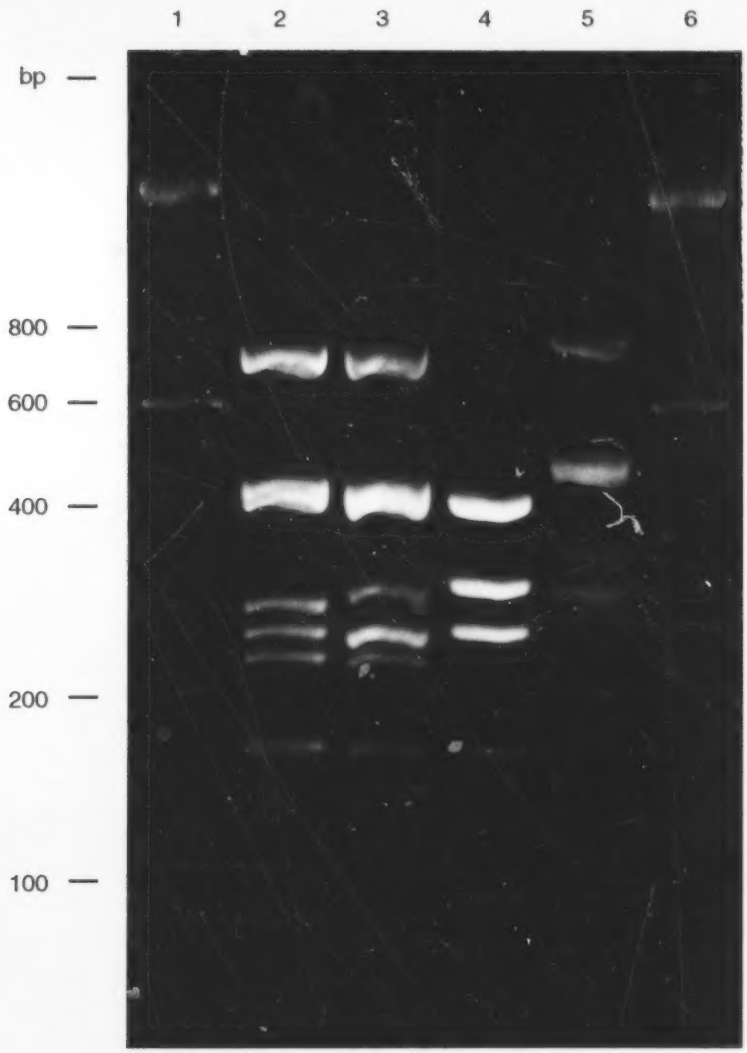


Figure 8. DNA fragments used in the plasmid DNA studies. *Hae* III digestion products were analyzed by electrophoresis on a 3% (w/v) NuSieve® gel containing 0.5 ug/ml ethidium bromide in 1X TAE electrophoresis buffer. Lanes 1 and 6: 100 bp molecular size standard; lane 2: 2 ug digested pHAS; lane 3: 2 ug digested pHAS+I; lane 4: 1.5 ug digested insert; lane 5: 1.8 ug pHAS+I+L. Molecular size standards are shown on the left.

171, 151, 142, 139, 127, 125, 102, 80, 54, 33, 18 and 11 bp, and those above 100 bp were visible on a 3% (w/v) NuSieve^R gel. The six extra bands in lane 3 (309, 262,171,139, 127 and 33 bp) are insert fragments unique to the pHAS+I target population. As a positive control, the complete insert was removed from the plasmid by *Sma* I/*Hin*d III restriction enzyme digestion and gel purification. Digestion of this insert with *Hae*III produced 8 fragments: 420, 309, 262, 171, 139, 127, 122 and 33 bp, and those above 125 bp are visible in Figure 8, lane 4. Six of these fragments are identical to the unique insert fragments in lane 3. The two extra fragments in this sample are the two ends of the insert each connected to part of the multiple cloning site, and are 420 and 122 bp in length.

The DNA samples shown in Figure 8 were used in the following experiments. Southern membranes were prepared using the DNA samples shown in lanes 2, 3 and 4. Three μg of pHAS fragments, 3 μg of pHAS+I fragments and 2 μg of insert fragments were electrophoresed through a 3% (w/v) NuSieve^R gel and transferred to a positively charged nylon membrane (Sambrook *et al* 1989). Figure 8, lane 2 also represents pHAS DNA fragments which were biotin labeled and became the driver DNA in the subtraction hybridization reactions. The fragments in Figure 8, lane 5 represent the pHAS+I fragments that have been ligated to the synthetic oligonucleotides (pHAS+I+L), and are ready to be DIG labeled and become the target DNA in the subtraction procedure.

5.1.2 Biotin labeling and capture of biotinylated fragments

Photoactivatable biotin was used to label the digested pHAS DNA (driver DNA population). This analogue of biotin was first developed by Forster *et al* (1985), and has been extensively used to quickly and reliably prepare biotin labeled DNA. The labeling procedure involved exposing the reaction mixture to a strong visible light resulting in a covalent bonding of biotin to the nucleic acids (Photoprobe^R biotin product information insert, Vector Laboratories, Burlingame, California). Many reported biotinylations have used as few as one or two labeling reactions (Straus *et al* 1990; Rubenstein *et al* 1990; van Klaveren *et al* 1994). It is important that each individual DNA strand have at least one biotin molecule, since after the hybridization step, driver DNA fragments must be completely removed from the solution. Excessive biotinylation is undesirable. Too many biotin residues attached to each fragment may increase steric hindrance during the hybridization step as well as increase the amount of streptavidin coated beads required to bind the biotinylated DNA fragments and remove them from solution.

Figure 9A shows the results of the biotinylation experiments. Four μg of driver DNA fragments (pHAS) were repeatedly labeled with Photoprobe^R biotin, and a 1 μg sample was analysed after each cycle. Streptavidin coated magnetic beads were added to the samples and bound to the biotinylated fragments. A magnet attracted these fragments, leaving unlabeled DNA fragments in solution. The samples were analysed as described in Figure 9A. The results show that three biotin labeling cycles were required before all detectable fragments were

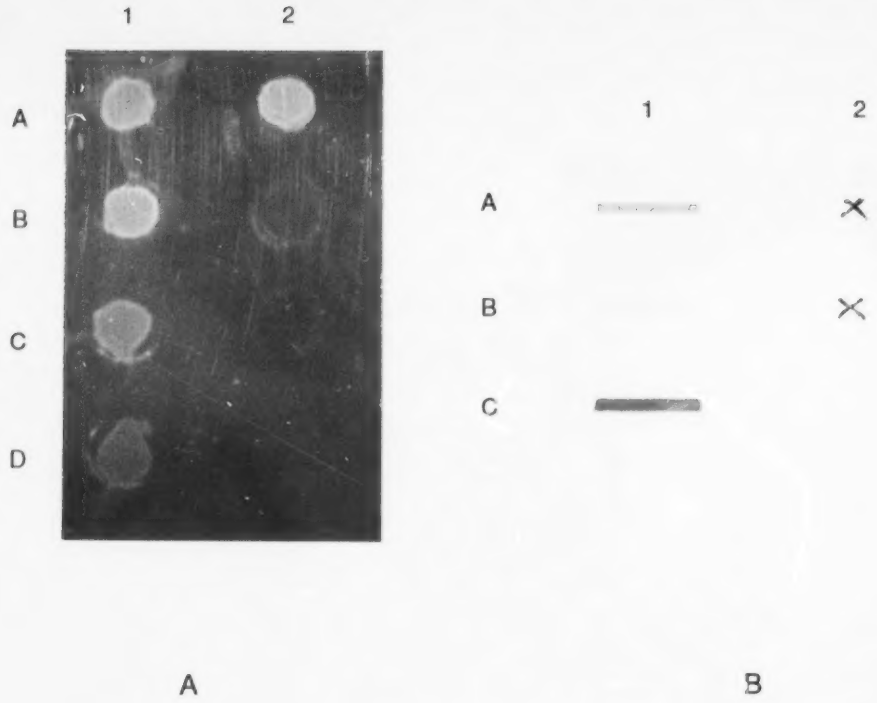


Figure 9. Evaluation of biotinylation. 9A Gel dot blot. Samples (1 ug) of biotinylated pHAS fragments before and after the capture treatment were dotted onto a 1% (w/v) agarose gel containing 1 ug/ml ethidium bromide. Position A1: biotinylated pHAS, positive control; A2: lambda DNA, negative control; B1,C1 and D1: before capture of biotinylated fragments following one, two and three biotinylations, respectively; B2, C2, D2: after capture of biotinylated fragments following one, two and three biotinylations respectively.
9B Slot blot. The final biotinylation samples (1 ug) before and after the capture treatment were attached to a nylon membrane and biotin detected. Position A1: biotinylated pHAS, positive control; B1: lambda DNA, negative control; C1: biotinylated pHAS labeled four times, before capture treatment; C2: biotinylated pHAS labeled four times, after capture treatment .

labeled and removed by the magnetic beads. A fourth biotinylation cycle was performed to maximize fragment labeling. Based on these results, 50 μg of pHAS fragments was labeled via 4 biotinylation cycles, producing driver DNA ready for the hybridization step.

Following the four biotin labeling cycles, a sample of the biotinylated pHAS DNA was tested to determine the quantity of streptavidin coated magnetic beads required to capture all detectable fragments. Four μg of biotinylated pHAS DNA required two 30 minute treatments with 1 mg of washed beads. Comparison of the biotin signal in Figure 9B between slot B1 (before capture) and B2 (after capture) shows the complete removal of detectable biotinylated DNA fragments.

5.1.3 Linker ligation

Two synthetic oligonucleotides were designed to anneal together and form a double stranded linker able to ligate to the *Hae* III digested blunt target DNA fragment ends. Once ligated, these linkers provided a common DNA sequence at each fragment end, regardless of the original fragment sequences. This known sequence could act as a priming site for labeling the DNA or for PCR amplification. Within the linker sequences are restriction enzyme sites which become valuable if future cloning steps are considered. Another design characteristic is the non-cohesive sticky ends. During the ligation reaction, these sticky ends do not have the opportunity to ligate together to form long concatemers of DNA. The noncohesive ends increase the efficiency of the

ligation reaction.

Referring to Figure 8, success of the linker ligation reaction was confirmed by comparison of the unligated fragments (lane 3) with the ligation products (lane 5). The visible fragments in lane 5 migrated at a higher molecular weight than their homologous fragments in lane 3. The similarity in banding patterns seen in lanes 3 and 5 indicates that the majority of fragments in lane 5 contain an extra 40 bp due to a linker ligated to each end and are referred to as pHAS+I+L.

The successful ligation seen in Figure 8 indicates that the preceding kinase reaction and oligonucleotide annealing steps were also successful.

5.1.4 DIG labeling

As mentioned in section 5.1.3, the oligonucleotide linkers provide a common DNA sequence at each end of the target DNA fragments. The 3' linker (CL-02) was the priming site for the digoxigenin (DIG) labeling reaction in which DIG-11-dUTP nucleotides were incorporated into the newly synthesized DNA strands. The DIG labeling of the pHAS+I+L target fragments was verified by comparing the signal generated by freshly labeled fragments with that generated by labeled control DNA provided in the labeling kit. The product insert states that 1 μg of template DNA results in 780 ng of DIG labeled fragments. The signal from the purified fragments was detected down to 10 pg.

In other studies, the ligation and labeling steps have been performed either before or after the subtractive hybridization step (Hakvoort *et al* 1994;

Plum *et al* 1994; Kikuta-Oshima *et al* 1994; Straus *et al* 1990). For this plasmid model system, the pHAS+I+L target fragments were DIG labeled prior to the hybridization step in order to detect any resulting fragments immediately following the subtractive hybridization step.

5.1.5 Subtractive hybridization and Southern analysis

Refering to figure 4, the two DNA populations were combined in a 10:1 ratio of biotinylated driver DNA: DIG labeled target DNA, denatured at 95°C and allowed to rehybridize overnight. The biotin labeled fragments were removed using the streptavidin coated magnetic beads, leaving reannealed target DNA fragments available for analysis.

The majority of the product was put back through the subtraction steps by the addition of fresh driver DNA. The remainder of each subtraction product was used to probe Southern membranes containing three lanes of denatured DNA: *Hae* III digested pHAS fragments, *Hae* III digested pHAS+I fragments and *Hae* III digested insert fragments. Figure 10 gives the Southern hybridization results. The control probe was 100 ng of DIG labeled target DNA, before the subtraction steps, and it found complementary sequences in all three lanes (Figure 10A). After the first, second and third subtraction (Figure 10A, 10B and 10C) the DIG signal in lane 1 began to decrease while the positive lane 3 signal remained strong. After 4 subtractions (Figure 10E), there was no visible signal in lane 1 even though the signal in lane 2 and 3 was so strong that the final individual bands could not be differentiated. This indicates that the final probe contains

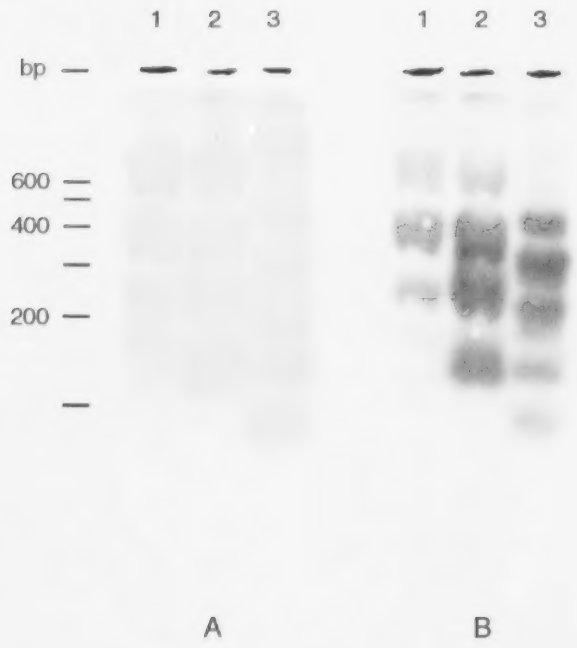
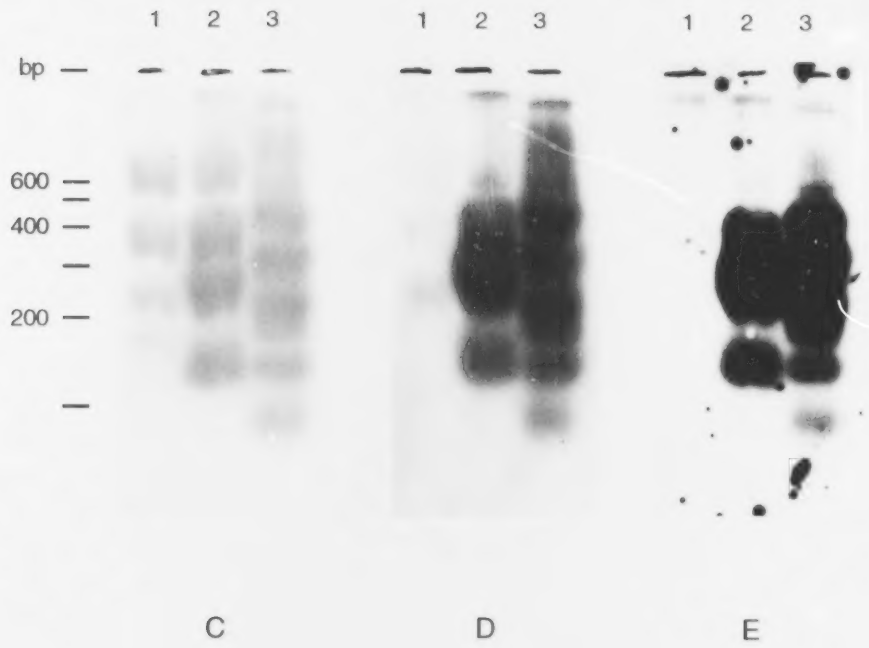


Figure 10. Southern blot analysis of plasmid fragments. A portion of each subtraction product was used to probe *Hae* III digests of pHAS (lane 1), pHAS+I (lane 2) and insert (lane 3) DNA. The probes were (A) DIG labeled pHAS+I before subtraction; (B) after one subtraction; (C) after two subtractions; (D) after three subtractions; and (E) after four subtractions. Molecular size standards are shown on the left.


1 2 3
1 2 3
1 2 3
bp
600
400
200
C
D
E

DIG labeled insert fragments unique to the pHAS+I target population.

These autoradiographs demonstrate the success of this subtraction procedure and its ability to select for nucleic acid segments unique to one population when the two populations are 65% homologous. They also show that fragments from 50 to 400 bp can be isolated using this method.

5.2 Mycobacterial study

The focus of the mycobacterial study was to detect unique *M bovis* DNA fragments by applying the subtractive hybridization techniques established during the plasmid study. A unique *M bovis* fragment could act as the basis for improved diagnostic testing and benefit both human and animal health programs since *M bovis* causes the same disease in both populations. As well, a unique *M bovis* fragment may improve epidemiological tracing and treatment of infected individuals.

Laboratory cultures of *M tuberculosis* and *M bovis* were provided by the mycobacterial lab of the Provincial Laboratory of Public Health, Edmonton, Alberta, and the biochemical test results for each strain are listed in Table 1. The *M tuberculosis* cells provided the driver DNA for the subtractive hybridization scheme and the *M bovis* DNA was the basis for the target DNA population.

	Mycobacterium tuberculosis	*Mycobacterium bovis*
Source	ATCC 25177	Isolate #92-MB21046
Pigmentation in Light	Buff	Buff
Pigmentation in Dark	Buff	Buff
Niacin Test	+	-
Nitrate Reduction	+	-
Catalase - 68°C	-	-
Catalase - SQ*	<45 mm	<45 mm
Urease	+	+
Pyrazinamidase	+	-
Nicotinamidase	+	-
Growth on TCH	ND	-
Growth on semi-solid media	aerobic	ND

* - SemiQuantitative
ND - Not determined

Table 1. Biochemical test results for the mycobacterial strains used in this study.

5.2.1 DNA isolation

The mycobacterial DNA isolation procedure was adapted from the procedure used by the Research and Development section of the Provincial Laboratory of Public Health (Edmonton, Alberta). The initial step where the cells were embedded in agarose blocks protected the DNA from shearing while the mycolic acid wall and cell membrane were digested. Once precipitated, intact genomic DNA can be difficult to redissolve, so one alteration in the procedure was to ensure that the DNA was never precipitated. One drawback was the 5 day length of the procedure, including one 24 hour incubation and one 48 hour incubation. However, since the resulting DNA sample exhibited very little shearing as measured by agarose gel electrophoresis and risk to personnel is minimized during the procedure, this added time was worthwhile to prepare the high quality DNA required for subtractive hybridization.

As described in section 4.5.1, following the first incubation step, complete inactivation of the cellular mixture was verified by inoculating a sample onto LJ slants. For all *M tuberculosis* and *M bovis* isolations, there was no visible growth after the 6 week incubations, indicating complete sterilization of all samples.

The *M tuberculosis* genomic DNA was isolated from the avirulent $H_{37}Ra$ laboratory strain (ATCC 25177). These cells grew well on LJ slants, exhibiting a crumbly colony texture at 2-3 weeks of age. A total of 2400 μg of *M tuberculosis* DNA was purified, and the overall average yield was 48 μg DNA per 100 mg cells (wet weight). The absorbance ratio of the final sample was 1.89 (A_{260}/A_{280}) and the DNA was stored in sterile water at 4°C.

The target DNA was isolated from a laboratory maintained *M bovis* culture which originated as a human clinical isolate (#92-MB21046) and grew relatively slowly. The cultures were incubated for 6 weeks and the DNA was isolated, yielding a total of 131 μg. The overall average yield was 10.7 μg DNA per 100 mg cells (wet weight) and the absorbance ratios of the three final samples were between 1.87 and 2.00 (A_{260}/A_{280}).

5.2.2 Partial digests

Complete digestion of the mycobacterial DNA samples with the *Hae* III restriction endonuclease resulted in fragments smaller than 400 bp (as determined by gel electrophoresis). Partial digestions are designed to cut the DNA at random restriction enzyme sites rather than at each site, resulting in increased diversity in the fragment sequences. For this mycobacterial study, the most consistent partial digestions were obtained when the reaction mixture contained 0.1 μg/μl genomic DNA and 0.15 U *Hae* III/ μg of DNA. The partially digested products were immediately electrophoresed through a LMP agarose gel. For the *M tuberculosis* partial digests, a block containing the fragments between 800 and 3,000 bp was excised and the DNA purified as described in section 4.5.2. Partial digestion of 910 μg of *M tuberculosis* genomic DNA resulted in 113 μg of purified fragments. The spectrophotometric absorbance ratio for the final sample was 1.75 (A_{260}/A_{280}). For the *M bovis* partial digests, a block containing the fragments between 300 to 800 bp was excised. Again, the DNA was purified as described in section 4.5.2. Partial digestion of 95 μg of *M bovis* genomic DNA

resulted in 20 μg of purified fragments with an absorbance ratio of 1.55 (A_{260}/A_{280}).

5.2.3 Biotinylation and capture of labeled *M tuberculosis* fragments

Biotinylation of the *M tuberculosis* fragments followed the procedure established during the pHAS plasmid study (section 5.1.2). Two reaction tubes each contained 50 μg of partially digested *M tuberculosis* fragments. The DNA in each tube went through 4 biotin labeling cycles. The resulting biotinylated samples were combined and the concentration was considered to be 0.5 μg/μl (based on the amount of starting DNA).

An important part of the subtraction scheme is to ensure the complete removal of all biotinylated fragments following the hybridization step. These fragments represent DNA that is common to both populations and any strands remaining in solution will interfere with the detection of unique fragments. As with the plasmid work, a sample of the biotinylated *M tuberculosis* DNA was tested to determine the quantity of streptavidin coated magnetic beads required to capture all detectable fragments. Five μg of this sample required five 30 minute binding cycles with 0.85 mg washed beads per treatment to remove all detectable biotinylated DNA from the solution.

5.2.4 Linker ligation

The two complementary oligonucleotides shown in Figure 5 anneal to produce linkers. These were ligated to the *Hae* III digested blunt *M bovis*

fragment ends and the ligation reaction was based on the conditions established during the plasmid study (section 5.1.3). The one difference was the 100X molar excess of linkers (rather than 10X). In the plasmid study, successful ligation was evident by comparing the migration of the fragments without and with linkers through a 3% (w/v) NuSieve^R gel (Figure 8, lane 3 versus lane 5). Since the *M bovis* fragments were partially digested, they ranged in length from 300 to 800 bp, producing a smear of DNA when electrophoresed through a 2% (w/v) agarose gel and the increase in size following the linker ligation was difficult to determine. To ensure proper linker ligation, the molar ratio of linkers to fragments was increased.

Following the ligation reaction, the fragments were gel and Centricon^R-100 purified (as described in section 4.3.2), resulting in 3.4 μg of target DNA fragments in sterile water with an absorbance ration of 1.61 (A_{260}/A_{280}).

5.2.5 Analysis of subtraction products

5.2.5.1 PCR Amplification

The detailed subtraction steps are described in section 4.5.3. In summary, the driver DNA (biotinylated *M tuberculosis*) and target DNA (linker ligated *M bovis*) were combined in a 10:1 ratio, denatured and allowed to hybridize overnight. The biotinylated fragments were removed from the reaction products, leaving the annealed target fragments in solution. A representative sample of this product was saved for analysis, and the remainder was

resubtracted, starting with the addition of driver DNA.

The DNA in the representative samples taken following each subtraction cycle was PCR amplified. The reactions were designed to amplify the *M bovis* target fragments based on their short oligonucleotide linker sequences. These linkers were DNA segments specific to the ends of each *M bovis* fragment and the oligonucleotide CL-01 was used as one primer that would anneal to the 5' end of each fragment and prime the PCR. The products seen in Figure 11 show the amplification of various template DNA samples. Refering to Figure 11, lane 2, 10 ng of biotinylated *M tuberculosis* fragments was used as template DNA resulting in four faint bands at approximately 650, 480, 450 and 350 bp. Including 1 μl of this PCR product in a second PCR amplified these same bands, along with two other bands at approximately 550 and 400 bp (lane 3). These PCR results indicate that the CL-01 primer was finding complementary sites within the *M tuberculosis* genome. The objective of the study was to specifically amplify remaining *M bovis* fragments. These PCR results are undesirable since they indicate that the subtraction product still contains *M tuberculosis* DNA. These bands could result from either incomplete biotinylation prior to subtraction, or from incomplete removal of all biotinylated fragments following the subtraction step, resulting in *M tuberculosis* fragments available for PCR amplification.

For future work, there are two options to decrease this non-specific amplification. Slight changes to the CL-01 sequence could make the primer specific for the ligated linkers and decrease the binding of primer to complementary sites within the *M tuberculosis* DNA. Cleaner preparation of the biotinylated driver population may improve the capture of biotinylated fragments

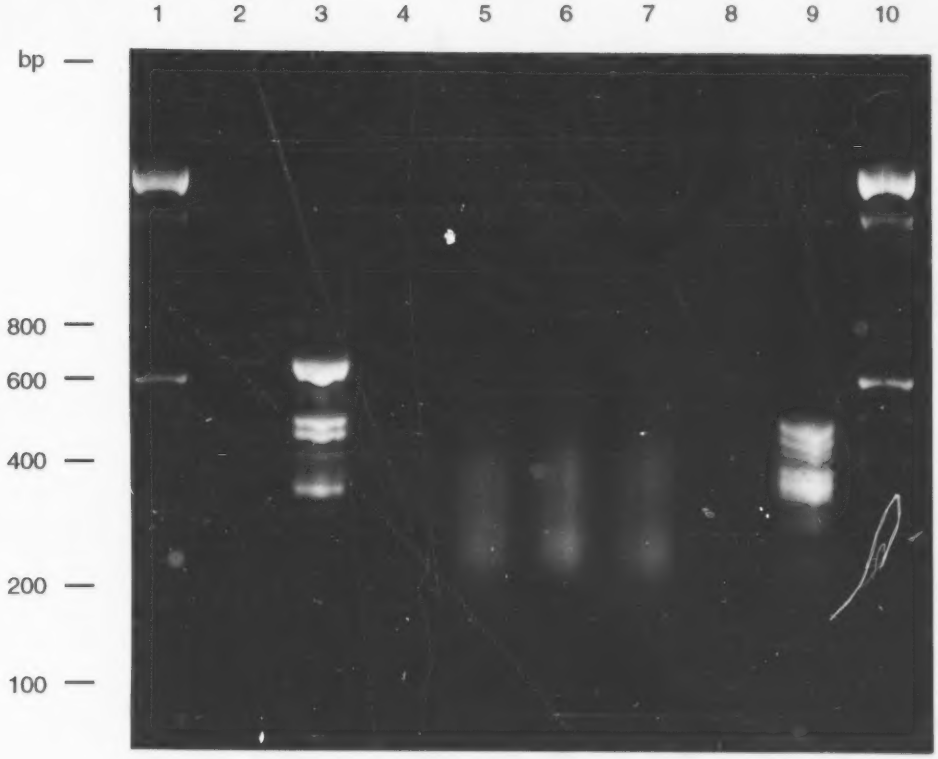


Figure 11. Comparison of PCR products generated from template DNA samples. Reaction products (10 ul) were analyzed by electrophoresis on a 2 % (w/v) agarose gel containing 0.5 ug/ml ethidium bromide in 1X TBE electrophoresis buffer. Lanes 1 and 10: 100 bp molecular size standard. Template DNA is: lane 2: biotinylated *Mycobacterium tuberculosis* partial digest fragments; lane 3: reamplification of 1 ul of PCR product shown in lane 2; lane 4: *Mycobacterium bovis* partial digest fragments; lane 5: *Mycobacterium bovis* fragments after linker ligation (before subtraction); lane 6: 0.1 volumes of the supernatant after one subtraction; lane 7: 0.11 volumes of the supernatant after two subtractions; lane 8: 0.125 volumes of the supernatant after three subtractions; lane 9: reamplification of 1 ul of PCR product shown in lane 8. Molecular size standards are shown on the left.

following the subtraction steps and decrease the amount of remaining driver fragments.

Template DNA for lane 4 (Figure 11) was 10 ng of unligated *M bovis* DNA fragments. A faint smear shows amplification products between approximately 400 and 200 bp. This indicates the CL-01 primer designed for the linker sequence was finding complementary sequences within the *M bovis* genome and priming amplification. Template DNA for lane 5 was 11 ng of ligated *M bovis* fragments. A strong smear of products can be seen between about 800 and 150 bp. Comparison of the products in lane 4 and 5 indicates the CL-01 primer was binding to the ligated linker sequence and priming the PCR, demonstrating the success of the linker ligation. The products seen in lane 5 also provides a PCR fragment profile before the subtraction procedure. The darkest area is centered around the 330 bp size indicating that the reaction selected for smaller fragments which may amplify at a slightly higher efficiency. This does not necessarily mean that the larger fragments are absent, they may just be overshadowed by the more prominent bands.

Template DNA for the reactions shown in Figure 11, lanes 6, 7 and 8 was a sample of the supernatant remaining after the capture of biotinylated fragments from the first, second and third subtraction reaction, respectively. Lanes 6 and 7 show a smear of reaction products from approximately 700 to 200 bp with fewer products at 300 bp. Lane 8 shows three very faint bands at approximately 500, 380 and 350 bp. Results from the model system indicated that four subtractions at a 10:1 ratio were needed to completely remove the common sequences. However, the PCR products from the third subtraction were barely visible (Lane

8), indicating the scarcity of *M bovis* DNA remaining after the three subtractions and these products were further studied.

Referring to Figure 11, lane 9, reamplification of 1 μl of the PCR product in lane 8 produced the same sized fragments as in lane 8 (500, 380 and 350 bp) along with 3 additional strong bands at approximately 450, 420 and 330 bp (lane 9). Since the pattern of these fragment sizes differs from the pattern in lane 3, unique fragments may have been amplified, and this sample was analysed further.

5.2.5.2 Purification of individual fragments

The DNA fragments seen in Figure 11, lane 9 were individually purified as described in section 4.5.6 (including Figure 7). Figure 12 shows the resulting PCR products. Lane 2 is the complete sample, identical to the sample in Figure 11, lane 9. The prominent bands in this lane may not be the only DNA fragments in the sample. These are the most abundant fragments and will exponentially amplify to a higher quantity compared to less abundant fragments. The fainter bands may be the most important, since they reflect rarer DNA templates. Thus, the complete lane of PCR products was divided into 4 blocks and the DNA in each block was further amplified. The PCR products amplified from each block are shown in lanes 3 to 6.

Amplification of the gel block #1 (between 800 and 480 bp) produced 2 bands of interest (lane 3, fragment 1A and 1B), amplification of block #2 (from 480 to 370 bp) produced 3 bands of interest (lane 4, fragment 2A, 2B and 2C),

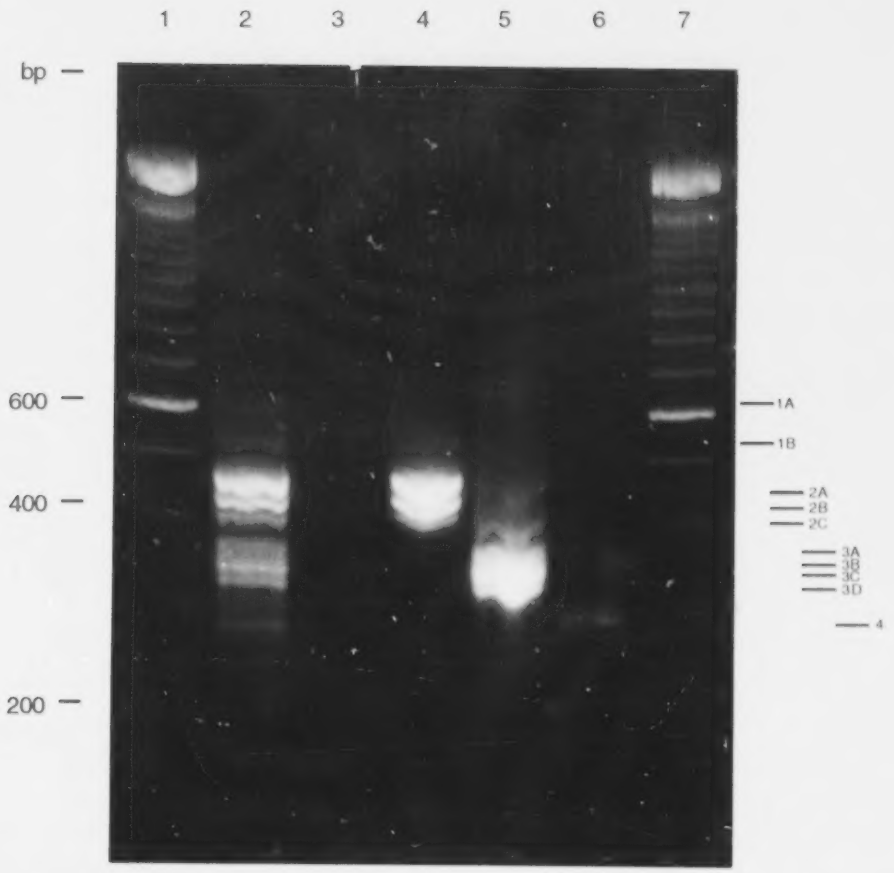


Figure 12. Amplification of DNA from the subtraction procedure. PCR products were analyzed by electrophoresis on a 3% (w/v) NuSieve® gel containing 0.5 ug/ml ethidium bromide in 1X TAE electrophoresis buffer. Lane 1 and 7: 100 bp molecular size standard; lane 2: 10 ul of second PCR product after three subtractions; lane 3: 20 ul amplified from block #1; lane 4: 10 ul amplified from block #2; lane 5: 10 ul amplified from block #3; lane 6: 20 ul amplified from block #4. Molecular size standards are shown on the left.

amplification of block #3 (between 370 and 300 bp) produced 4 bands of interest (lane 5, fragment 3A, 3B, 3C and 3D) and amplification of the fourth block (300 to 270 bp) produced one additional band (lane 6, fragment 4). As detailed in Figure 7, a total of 10 bands were excised and individually labeled with DIG.

5.2.5.3 Southern hybridization experiments

DIG labeled probes generated from the 10 individual fragments were used to screen a Southern membrane. The membrane was prepared as explained in section 4.5.7 and contained 3 lanes of denatured DNA samples. The first lane was the *Hind* III digested lambda marker. The second lane contained 5 μg of *Eco*R I digested *M tuberculosis* DNA and the third lane contained 5 μg of *Eco*R I digested *M bovis* DNA. Figure 13 displays the Southern hybridization results. In all these autoradiographs the marker lane has been removed since there was no visible hybridization between any of the probes and the DNA in this lane. For the control probe (Figure 13A) *M bovis* target DNA fragments were DIG labeled via PCR using the CL-01 oligonucleotide which bound to the linker sequence and primed the labeling reaction. This probe produced a strong signal throughout both lanes and the two membrane bound species were indistinguishable. Fragment 1A (Figure 13B) identified one very similar sequence in both DNA samples just below the 4.3 Kbp size marker, indicating that the probe represents a fragment of DNA that is common to both mycobacterial species. The probe sequence could have originated from *M bovis* DNA that rehybridized to its own complementary sequence rather than a complementary *M tuberculosis* sequence

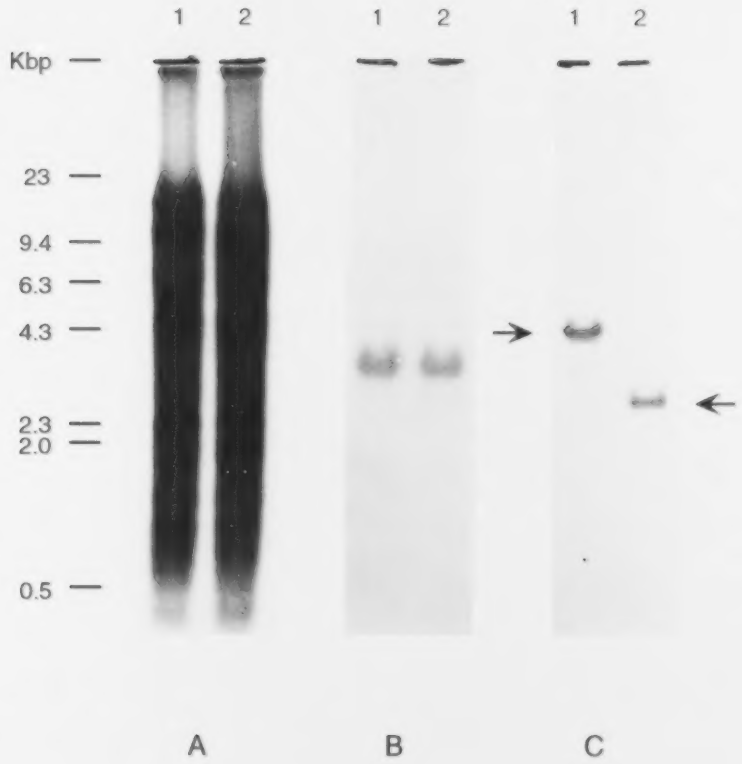
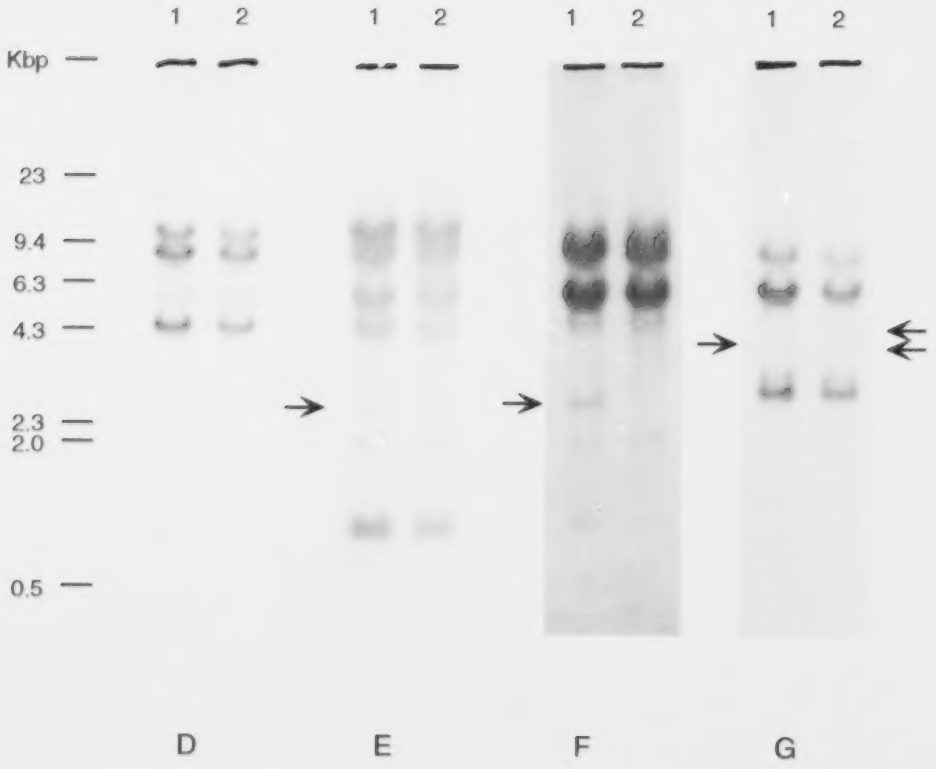
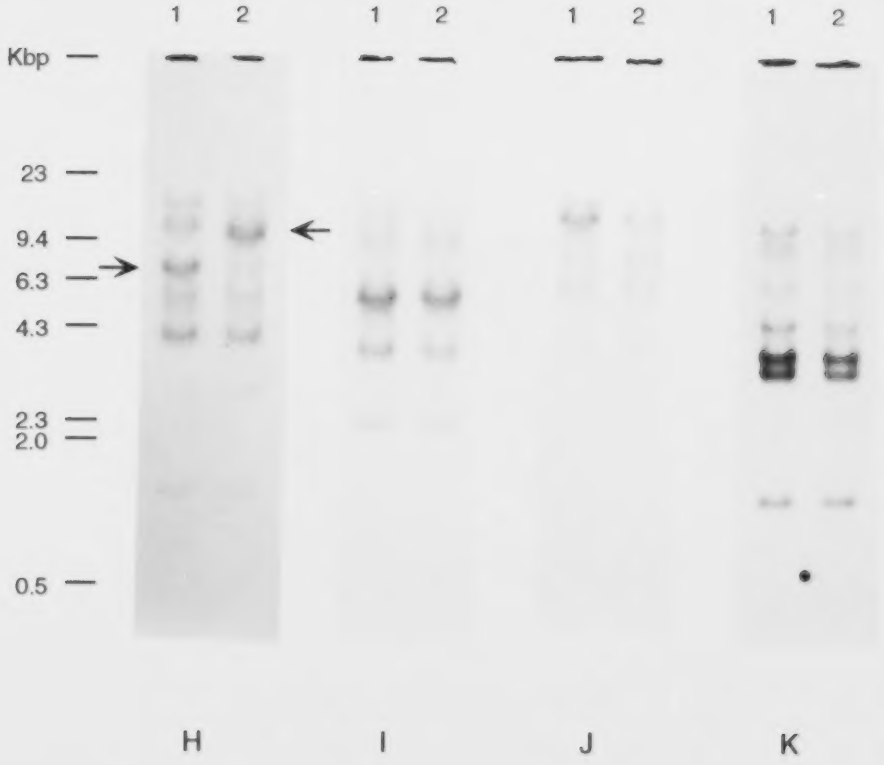


Figure 13. Southern bot analysis of subtraction products. DIG labeled PCR fragments were generated from DNA remaining after three subtractions. These fragments were used to probe *Eco*R I digests of *Mycobacterium tuberculosis* (lane 1) and *Mycobacterium bovis* (lane 2) genomic DNA. The probes were (A) DIG labeled *Mycobacterium bovis* before subtraction; (B) fragment 1A; (C) fragment 1B; (D) fragment 2A; (E) fragment 2B; (F) fragment 2C; (G) fragment 3A ; (H) fragment 3B; (I) fragment 3C; (J) fragment 3D; (K) fragment 4. Molecular size standards are shown on the left and arrows indicate significant bands.


1
2
1
2
1
2
1
2
Kbp
23
9.4
6.3
4.3
2.3
2.0
0.5
D
E
F
G


1
2
1
2
1
2
1
2
Kbp
23
9.4
6.3
4.3
2.3
2.0
0.5
H
I
J
K

during the subtractive hybridization step, or it could have originated from *M tuberculosis* DNA that was not completely removed following the subtraction step.

Fragment 1B (Figure 13C) gave one strong signal in both lanes, yet at two different sizes in the *Eco*R I digests. In the *M tuberculosis* lane the band is at about 4.0 Kbp and in the *M bovis* lane the band is near 2.5 Kbp. This indicates the probe is not a unique *M bovis* sequence, but it detects different sized genomic DNA fragments bound to the membrane, indicating a difference in the *Eco*R I recognition site. This offers an interesting opportunity for the design of a clinically useful diagnostic test. The difference could be a single base change in the *Eco*R I site in the *M tuberculosis* sequence, resulting in a DNA fragment that is not cut and runs as a larger fragment in the gel. In a diagnostic test, PCR primers flanking this *Eco*R I site would amplify genomic DNA from each species. A subsequent restriction digest using *Eco*R I would result in two fragments with *M bovis*, but leave the PCR product intact when the original sample was *M tuberculosis*. PCR primers could also be designed to bind at the species specific *Eco*R I site, producing a product in one species while not binding properly in the other species. To further investigate these opportunities, *M tuberculosis* and *M bovis* genomic libraries could be screened with this probe and the sequences of the positive clones may identify the specific differences.

The three fragments isolated from block 2 (Fragments 2A, 2B and 2C) produced multiple banding patterns in autoradiographs D, E and F (Figure 13). The patterns are very similar, with one interesting faint band in Lane 1 of autoradiograph E (Fragment 2B) and a similar band in Lane 1 of autoradiograph F (Fragment 2C). These are interesting bands, considering the subtraction steps

were designed to select for *M bovis* fragments. There may be a corresponding band elsewhere in Lane 2 that is hidden by the more prominent bands. Or, the band may be the result of incomplete *Eco*R I digestion of the genomic *M tuberculosis* DNA. The multiple banding patterns seen in all three of these autoradiographs (D, E and F) show the importance of isolating individual fragments from the sample of subtraction products electrophoresed through the initial agarose gel. It is possible that two subtraction products could be the same size, even though they have different sequences and bind to separate complementary bands on the Southern blot. Another consideration is that these probes code for a section of a mycobacterial insertion sequence. For future study, these three probes offer the least potential, considering the number of bands and the similarities in the banding patterns.

Four probes were isolated from block 3 (Fragments 3A, 3B, 3C and 3D). As with the probes purified from block 2, multiple banding patterns are evident. Referring to autorad G, Fragment 3A produced an interesting faint pattern near the 4.3 Kbp size. In the *M tuberculosis* lane, there is one band just below the marker size. In the *M bovis* lane, a comparable band is seen, with another individual band just above the 4.3 Kbp size. This probe could be used to detect positive clones in subgenomic libraries (5000 to 3500 bp) made from each species. The positive *M bovis* clones may contain an extra segment of DNA. Of all the probes investigated, Fragment 3B (Figure 13H) offers the best potential for future work. There is one set of corresponding bands at the 4.3 Kbp size in each lane, and the interesting set is up above the 7 Kbp size. The strongest band is in the *M bovis* lane (Lane 2) just above 9.4 Kbp. The corresponding band appears to be in Lane

1 near 7 Kbp. In addition to the size difference, there is a difference in the band intensity, indicating that more of the probe is binding to the *M bovis* fragments than the *M tuberculosis* fragments. Increasing the stringency of the hybridization or of the washes may decrease the signal from the remaining bands and maintain the intensity of this *M bovis* signal. To investigate a sequence difference, this probe could screen complete genomic libraries from each species. Approximately 50% of the strong positive clones will correspond to the two fragments of interest. Since the other bands appear at corresponding sizes, their sequences may be very similar, if not identical, and they could be screened out by sequence comparison. Screening of subgenomic libraries covering the fragments above 5000 bp would produce positive clones corresponding only to the two bands of interest. Fragment 3C produced similar banding patterns in both lanes (Figure 13 I). Fragment 3D showed similar banding patterns in both lanes, however the darkest set of bands between 23 Kbp and 9.4 Kbp show a stronger signal in lane 1 than in lane 2 (Figure 13J). Fragment 4 identified complementary sequences in both lanes and the banding patterns are indistinguishable (Figure 13K). As with fragment 1A, these final three probes (Fragments 3C, 3D and 4) seem to represent DNA fragments which are common to both populations. Further investigation to determine sequences for these probes or their corresponding membrane bound fragment does not look promising.

5.3 Summary

The subtractive hybridization techniques established by the plasmid study

selected for 10 visible mycobacterial DNA fragments. Southern hybridization experiments indicate that four of these fragments offer the potential for identification of a sequence which will differentiate *Mycobacterium bovis* from *Mycobacterium tuberculosis*.

6.0 FUTURE DIRECTION

The research described in this report did not isolate a DNA fragment that proved to be unique to the *M bovis* organism. This is not a surprising outcome, considering the level of homology between the two organisms.

The model system based on the pHAS plasmid demonstrated the success of subtractive hybridization when two populations are relatively simple and 65% homologous. For future applications, there are two steps in the procedure that could be improved. Biotin labeling of the driver DNA population was involved and time-consuming. An alternate option to repeat labeling is described by Aasheim *et al* (1994). The driver fragments are end labeled with biotin, attached to the streptavidin-coated magnetic beads and the labeled strands purified from the mixture using a magnet. The resulting bead-biotin-DNA fragments make a pure, stable, reusable driver DNA population, which can be consistently removed from the hybridization mixture. Since most subtraction procedures require a large quantity of driver DNA, reusing the same subtracting fragments reduces the time and expense involved with purifying enough DNA, labeling the fragments and ensuring that each piece is acting as expected. This approach would also conserve on the amount of beads required to remove the biotinylated DNA hybrids and the time involved in determining that amount.

The second adjustment to the overall procedure should involve the oligonucleotide linkers. Changing the base sequence may identify a primer which does not anneal to the driver DNA population as successfully as the primer used in this study. This would reduce the amplification of common driver DNA,

increasing the chance that the amplified fragment is unique to the target population. The sequence should still incorporate certain restriction enzyme sites valuable for the subsequent cloning of interesting fragments and some time would be required to design primers which meet these two requirements.

The mycobacterial study suggests interesting areas that could be investigated further. Complete genomic *M tuberculosis* and *M bovis* libraries could be screened with fragments 1B and 3B. Up to half of the positive clones should exhibit a sequence difference between the two species which represents the different banding pattern seen in the autoradiographs. This difference could be used to design PCR primers specific for these two mycobacterial strains. For the remaining interesting fragments (2B and 3A), two subgenomic libraries are needed from each strain. Each set could be made simultaneously from genomic DNA digested with *Eco*R I since fragments of 2300 to 3500 bp, and 3500 to 5000 bp could be excised from an agarose gel and ligated into appropriate vectors for screening, sequencing and primer design.

These two mycobacterial strains are very similar and this characteristic adds to the difficulty of using subtractive hybridization to identify a unique sequence. The present study isolated ten fragments, suggesting the potential value of subtractive hybridization. The few modifications suggested would improve the efficiency of this procedure and enhance the investigation of known or suspected genetic differences between organisms.

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